THE DETERMINATION OF LINAGLIPTIN USING HPLC METHOD

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032.

In partial fulfillment of the requirements for the award of Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted

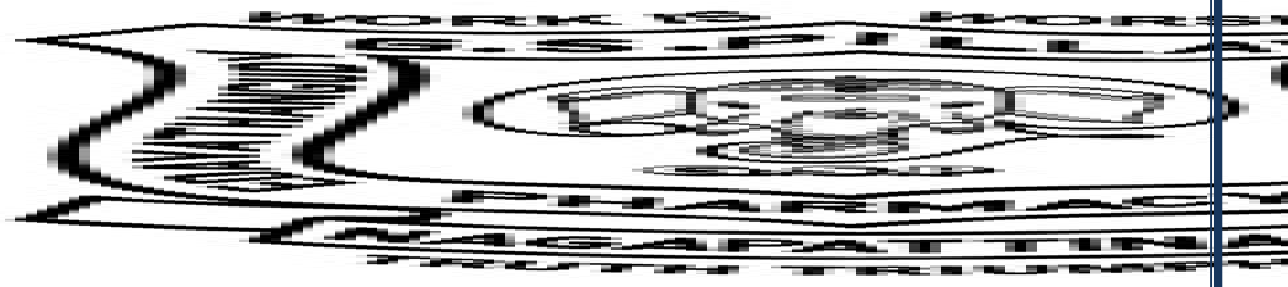
BY

VENKATA NARESH KUMAR.P

Reg.No.261230962

Under the guidance of

Mr. S. Justin Jayaraj, M.Pharm



DEPARTMENT OF PHARMACEUTICAL ANALYSIS,

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

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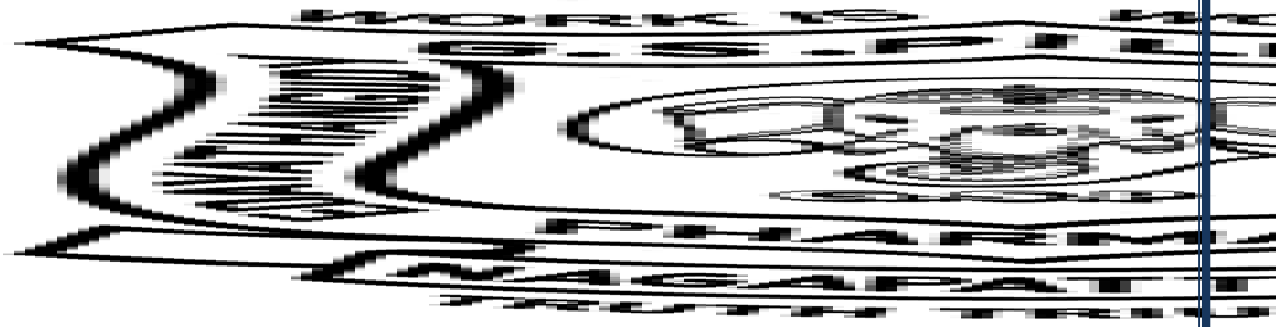

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CERTIFICATE

This is to certify that the dissertation entitled"The determination of linagliptin using hplc method"submitted by **VENKATA NARESH KUMAR.P** (Reg No:261230962) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in the Department of Pharmaceutical Analysis, Edayathangudy.G.SPillay College of Pharmacy during the academic year 2013-2014.

Place: Nagapattinam

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Place: Nagapattinam

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INDEX

1. INTRODUCTION

1.1. ANALYTICAL CHEMISTRY

Analytical chemistry is a branch of chemistry, which covers a series of subjects covering the science of chemical separation, identification, detection and measurement of a sample. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurement of the substances present in the bulk and pharmaceutical preparation. Qualitative identification provides information about the identity of an atomic, molecular or bimolecular species. Quantitative measurement provides numerical information as to the relative amounts of species.

METHODS OF DETECTING ANALYTES

1. Physical means

    - Mass
    - Color
    - Refractive index
    - Thermal conductivity

2. With electromagnetic radiation (Spectroscopy)

    - Absorption
    - Emission
    - Scattering

3. By an electric charge

    - Electrochemistry

    - Mass spectrometry

The newer methods for separating and determining chemical species are known collectively as instrumental methods of analysis. Most of the instrumental methods fit into one of the three following categories viz., spectroscopy, electrochemistry and chromatography.

ADVANTAGES OF INSTRUMENTAL METHODS

- Small samples can be used.
- High sensitivity is obtained.
- Measurements obtained are reliable.
- Determination is very fast.
- Even complex samples can be handled easily.

LIMITATIONS OF INSTRUMENTAL METHODS

- An initial calibration is required.
- Sensitivity and accuracy depends on the instrument.
- Cost of the equipment is large.
- Concentration range is limited.
- Specialized space is required.

1.2. PRINCIPLE TYPES OF CHEMICAL INSTRUMENTATION

SPECTROMETRIC TECHNIQUES

i. Ultraviolet and visible spectroscopy
ii. Fluorescence and phosphorescence spectroscopy
iii. Atomic spectroscopy
iv. Infrared spectroscopy
v. Raman spectroscopy
vi. X-Ray spectroscopy
vii. Radiochemical Techniques including activation analysis
viii. Nuclear Magnetic Resonance spectroscopy
ix. Electron Spin Resonance spectroscopy

ELECTROCHEMICAL TECHNIQUES

i. Potentiometry

ii. Voltametry
iii. Stripping Techniques
iv. Amperometric Techniques
v. Electrogravimetry
vi. Conductance Techniques

CHROMATOGRAPHIC TECHNIQUES

i. Gas Chromatography
ii. High Performance Liquid Chromatography
iii. High Performance Thin Layer Chromatography

MISCELLANEOUS TECHNIQUES

i. Thermal analysis
ii. Mass spectroscopy
iii. Kinetic Techniques

HYPHENATED TECHNIQUES

i. Gas Chromatography - Mass spectroscopy (GC-MS)
ii. Inductivity coupled plasma -Mass spectroscopy (ICP – MS)
iii. Gas Chromatography- Infrared spectroscopy (GC-IR)
iv. Mass spectrometry (MS-MS)

1.3 PRELIMINARY STUDIES

a) Determination of melting point.
b) Determination of wavelength of maximum absorption.

1.3.1. MELTING POINT:

A melting point can be used to identify a substance and to get an indication of its purity. The melting point (or freezing point) of a solid is the temperature at which the solid exists in equilibrium with its liquid state under an external pressure of one atmosphere. Both the melting point range (the interval between the beginning of liquefaction and complete liquefaction) and the temperature of complete liquefaction are valuable indicators of the purity of the solid compound. A pure crystalline organic compound usually possesses

a sharp melting point and it melts completely over a narrow temperature range of not more than 0.5-1.0ºC, provided good technique is followed. The presence of even small amounts of impurities usually produces a depression of the temperature at which melting is complete and usually produces a marked increase in the width of the melting point range.

Procedure: Fill a melting point capillary tube with the sample of linagliptin by thrusting the open end into the powder several times. In order to work the plug of solid material down to the sealed end of the capillary, tap the sealed end on the table. Repeat the procedure until the tube contains a 3 mm column of densely packed powder in the bottom. Place the capillary in the melting point apparatus through one of the side tubes so that the sealed end of the capillary is dipped in liquid parrafin.Gradually the temperature raises and substance in the capillary tube starts melting, note the temperature.

1.3.2. DETERMINATION OF WAVELENGTH OF MAXIMUM ABSORPTION:

In analyzing a new sample, a chemist first determines the sample's absorbance spectrum. The absorbance spectrum shows how the absorbance of light depends upon the wavelength of the light. The spectrum itself is a plot of absorbance vs wavelength and is characterized by the wavelength (λ_{max}) at which the absorbance is the greatest. The value of λ_{max} is important for several reasons. This wavelength is characteristic of each compound and provides information on the electronic structure of the analyte. In order to obtain the highest sensitivity and to minimize deviations from Beer's Law. Analytical measurements are made using light with a wavelength of λ_{max}.

Procedure: An appropriate concentration of sample solution (20µg/mL) was scanned to determine λ_{max} in UV-Visible spectrophotometer.

1.4ANALYTICAL METHOD DEVELOPMENT:

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the

existing procedure,comparative laboratory data including merits/demerits should be made available.

STEPS INVOLVED IN METHOD DEVELOPMENT:

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. All data to these studies must be recorded in laboratory notebook or an electronic database.

ANALYTE STANDARD CHARACTERIZATION:

1. All known information about the analyte and its structure is collected i.e., physical and chemical properties.
2. The standard analyte (100% purity) is obtained. Necessary arrangement is made for the proper storage(refrigerator,desiccators and freezer).

METHOD REQUIREMENTS:

The goals or requirements of the analytical method that need to be developed are defined below. The required detection limits, selectivity, linearity, range, accuracy, and precision are defined.

LITERATURE SEARCH AND PRIOR METHODOLOGY:

The literature for all types of information related to the analyte is surveyed, for synthesis physical and chemical properties, solubility, relevant analytical methods in books, periodicals, chemical manufacturers and regulatory agency compendia such as USP/ NF are reviewed. Chemical abstracts service (CAS) automated computerized literature searches are convenient for literature survey.

CHOOSING A METHOD:

Based on the information of analyte properties and methods reported literature method is developed. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.

If there are no prior methods for the analytein the literature, from analogy the compounds that are similar in structure and chemical properties are investigated and are worked out.

INSTRUMENTAL SETUP AND INITIAL STUDIES:

The required instrumentation is arranged, installed, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) is verified.

During the analytical method development analytical grade consumables (solvents, filters and gases) are used, and high quality columns and instruments are employed.

Theanalyte standard in a suitable solvent is prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample.

OPTIMIZATION

During optimization one parameter is changed at a time and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented in a lab notebook.

DOCUMENTATION OF ANALYTICAL FIGURES OF MERIT:

The originally determined analytical figures of merit are limit of quantification (LOQ), Limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

EVALUATION OF METHOD DEVELOPMENT WITH ACTUAL SAMPLES:

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

DETERMINATION OF PERCENT RECOVERY OF ACTUAL SAMPLE AND DEMONSTRATION OF QUANTITATIVE SAMPLE ANALYSIS:

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of the recovery (average, standard deviation)from sample to sample and whether recovery has been optimized or not has been shown. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty.

STABILITY-INDICATING METHOD DEVELOPMENT

A stability-indicating method is defined as an analytical method that accurately quantities the active ingredients without interference from degradation products,. A method that accurately quantititates significant degradant may also be considered stability-indicating. A proactive approach to developing a stability indicating HPLC method should involve forced degradation at the early stages of development with the key degradation samples used in the method development process.

If forced degradation studies are performed early, method development and identification of primary degradation products and unknown impurities can be run in parallel. Using this process a validated HPLC analytical assay mechanism of degradation and the impurity/degradant information for filing can all be generated without delays in the project timeline.

1.5. ANALYTICAL METHOD VALIDATION

According to the International Conference on Harmonisation (ICH) method validation can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting is predetermined specifications and quality characteristics". Method validation is an integral part of the method development it is the process by which a method is tested by the developer or user for reliability, accuracy and preciseness of its intended purpose and demonstrating that analytical procedure are suitable for their intended use that they support the identity, quality ,purity, and potency of the drug substances and drug products data thus generated become part of the method validation package submitted to Center for Drug Evaluation and Research (CDER).

Methods should be reproducible when used by other analysts, on other equivalent equipment, on other days or locations, and throughout the life of the drug product. Data

that are generated for acceptance, release, and stability or pharmacokinetic will only be trust worthy if the methods used to generate the data are reliable. The process of validation and method design also should be clearly in the development cycle before important data are generated. Validation should be on going in the form of re-validation with method changes.

Though many types of HPLC techniques are available the most commonly used method, the reversed phase HPLC with UV detection, is selected to illustrate the parameters for validation. The criteria for the validation of this technique can be extrapolated to other detection methods and chromatographic techniques. In this technique all the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection data evaluation.

Analytical parameters includes namely

I. System suitability
II. Accuracy
III. Precision
IV. Linearity
V. Specificity/selectivity
VI. Limit of Detection
VII. Limit of Quantification
VIII. Robustness

1. SYSTEM SUITABILITY:

According to USP system suitability tests are an integral part of chromatographic method. These tests are used to verify that the resolution and reproducibility of the system that are adequate for the analysis to be performed.The purpose of the system suitability test is to ensure that the complete testing system (including instrument, reagents, columns and analysts) is suitable for the intended application.

During the early stage of the method development process some of the more sophisticated system suitability tests may not be practical due to the lack of experience with the method. In this stage, usually a more “generic” approach is used. For example, evaluation of the tailing factor to check chromatographic suitability and replicate injections of the system suitability solution to check injection precision may be sufficient

for an HPLC assay. As the method matures more experience is acquired for this method, a more sophisticated system suitability test may be necessary.

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (% RSD of retention time and peak area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of system suitability "sample" that is a mixture of main components and expected by-products. The terms that should be measured and their recommended limits obtained from the analysis of the system suitability sample as per current ICH guidelines on "validation of chromatographic methods" were given below

Acceptance criteria:

Parameter	Recommendation
Capacity Factor (k)	The peak should be well-resolved from other peaks and the void volume , generally k >2.0
Repeatability	RSD≤2% for N≥5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Tailing factor(T)	T of ≤2
Theoretical plates (N)	N> 2000

2. ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found[15]. This is sometimes termed as "trueness". The different methods for analysis of accuracy are

i. Analyzing sample of known concentration and comparing the measured value to the true value. However, a well characterized sample (e.g. reference standard) must be used.
ii. Spiked – placebo (product matrix) recovery method: In this method, a known amount of pure active constituent is added to formulation blank sample that contains all other ingredients except active ingredient, resulting mixture is assayed, and the results obtained are compared with the expected result.

iii. Standard addition method: In this method initially a sample is assayed, then a known amount of pure active constituent is added to the sample, and the sample is again assayed. The difference between the results of the two assays is compared with the expected answer.

In both methods (spiked – placebo recovery and standard addition method), recovery is defined as the ratio of the observed result to the expected result and expressed as a percentage. The accuracy of a method may vary across the range of possible assay values and therefore must be determined at several different fortification levels. The accuracy should cover at least 3 concentrations (80,100, 120%) in the expected range. Accuracy may also be determined by comparing test results with those obtained using another validated test method.

Acceptance criteria:The mean % recovery at each experiment should not be less than 98.0% and not more than 102.0%.

3. PRECISION

The precision of an analytical procedure expresses the degree of closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Precision may be considered at three levels:

I. System precision (Repeatability)

II. Method precision (Reproducibility)

III. Intermediate precision (Ruggedness)

- **Repeatability**

Repeatability should be obtained when the analysis is carried out in laboratory by an operator using equipment over a relatively short time span. They must contain

a) A minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or

b) A minimum of 6 determinations at 100% of the test concentration.

- **Intermediate Precision**

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

- **Reproducibility**

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier.

The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

Acceptance criteria:% RSD of peaks areas should not be more than 2.0%

4. LINEARITY

The linearity of an analytical procedure is its ability within a given range to obtain test results which are directly proportional to the concentration of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance by dilution of a standard stock solution using the proposed procedure[(11)].

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be further evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. For the establishment of linearity, a minimum of 5 concentrations calibration curve is recommended.[(35)]

Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration of an analyte in a sample.

Acceptance criteria: Correlation coefficient should be not less than 0.999.

5.SPECIFICITY /SELECTIVITY:

The terms selectivity and specificity are often used interchangeably. According to International Conference on Harmonisation (ICH), the term specific generally refers to a method that produces a response for a single analyte only while the term selectivity refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.

The analyte should have no interference from other extraneous components and be well resolved from them. A representative HPLC chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte

6. DETECTION LIMIT

The limit of detection of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Based on the standard deviation of the response and the Slope the LOD may be expressed as

$$LOD = \frac{3.3\ \sigma}{S}$$

Where σ = The standard deviation of the response

S = The slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example

1. **Based on the standard deviation of the blank**

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

2. **Based on the calibration curve**

A specific calibration curve should be studied using samples containing an analyte in the range of detection limit. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

7. QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

Based on standard deviation of the response and of the slope, the Quantitation limit (QL) may be

expressed as

$$LOQ = \frac{10\,\sigma}{S}$$

where σ = The standard deviation of the response

S = The slope of the calibration curve

8. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters is established to ensure that the validity of the analytical procedure is maintained whenever used.

In the case of liquid chromatography, examples of typical variations are influence of variations of pH in a mobile phase, influence of variations in mobile phase composition, different columns (different lots and/or suppliers), temperature, flow rate.

9. SOLUTION STABILITY

To generate reproducible and reliable results, the samples, standards and reagents used for HPLC method must be stable for a reasonable time (e.g., one day, one week, one month, depending upon the need). Therefore, a few hours of standard and sample solution stability can be required even for short separation. When more than one sample is analyzed (multiple lots of one sample or samples from different storage conditions from a single lot), automated, overnight runs often are performed for better lab efficiency. Such practices add requirements for greater solution stability.

Samples and standards should be tested during a period of at least 24 h (depending on intended use), and component quantification should be determined by comparison with freshly prepared standards. For the assay method, the sample solutions, standard solutions and HPLC mobile phase should be stable for 24 h under defined storage conditions. Acceptable stability is <2% change in standard or sample response, relative to freshly prepared standards. The mobile phase is considered to have acceptable stability if aged

mobile phase produces equivalent chromatography (capacity factors, resolution or tailing factor) and the assay results are within 2% of the value obtained with fresh mobile phase.

1.6 FORCED DEGRADATION STUDIES

Forced degradation or stress testing is undertaken to demonstrate specificity when developing stability-indicating methods, particularly when little information is available about potential degradation products. These studies also provide information about the degradation pathways and degradation products that could form during storage. Forced degradation studies may help facilitate pharmaceutical development as well in areas such as formulation development, manufacturing, and packaging, in which knowledge of chemical behavior can be used to improve a drug product[(2)].

EXPERIMENTAL APPROACH TOOLS:

Forced degradation studies of Active Pharmaceutical Ingredient (API) and Drug Product (DP) include appropriate solid state and solution state stress conditions (like acidic/base hydrolysis, heat, oxidation and light exposure) in accordance with ICH guidelines. Forced degradation studies should be conducted whenever a stability indicating method is required. Studies may need to be repeated as methods, processes, or formulations change. The specified stress conditions should result in approximately 5-20% degradation of the API or represent a reasonable maximum condition achievable for the API. The stressed sample should be compared to the unstressed sample (control) and the appropriate blank. A compound may not necessarily degrade under a given stress condition. No further stressing is advised in these cases

1. ACID:

Typical acids include HCl or H_2SO_4 (0.1-1 mol/L solution).Studies should be carried out in the solution state. For certain APIs that are partially soluble or insoluble in the described acidic solution,addition of an appropriate co-solvent, or adjustment of solution pH in the acidic range may be required to achieve dissolution, or the APIs can be run as suspensions.Special attention to the API structure should be paid when choosing the

appropriate co-solvent (i.e. do not use alcohols for acidic conditions due to their reactivity).Dimethylsulfoxide (DMSO), acetic acid and propionic acid are useful for acidic conditions. Additionally, the sample may be heated for a defined time and temperature to accelerate degradation, depending on the API sensitivity to heat.

2. BASE:

Typical bases include NaOH, LiOH, or KOH (0.1- 1 mol/L). Studies should be carried out in the solution state. For certain API which are partially soluble or insoluble in the described basic solution, addition of an appropriate co-solvent, or adjustment of solution pH may be required to achieve dissolution, or the APIs can be run as suspensions. Additionally, the sample may be heated for a defined time and temperature to accelerate degradation depending on the API sensitivity to heat.

3. OXIDATION:

Oxidation can be carried out under an oxygen atmosphere or in the presence of peroxides. The use of oxygen is a more realistic model. Free radical initiator and peroxide will produce a primary oxidation, degradation products are observed on real-time stability. Therefore, free radical and/ or hydrogen peroxide condition based degradation are strongly recommended at all stages of development. For solution state stress conditions, dissolve the API utilizing an appropriate solvent, add 5-20 mol% of a free radical initiator at atmospheric pressure. To increase the solubility of oxygen in the solution, the reaction can be performed in a reaction vessel pressurized at 50-300 psi (pound per square inch) with molecular oxygen. Additionally the system is heated to accelerate degradation. The temperature depends on the free radical initiator selected. For peroxide conditions, hydrogen peroxide reagent (up to 3%) can be used. As previously indicated, the addition of an appropriate co-solvent may be necessary, depending on API solubility. Hydrogen peroxide stress testing can be useful in DP studies where hydrogen peroxide is an impurity in excepients. Solid- state stress conditions may be similarity investigated by placing the API in suitable closed containers filled with an oxygen headspace versus argon or nitrogen control headspace. Further, light can also affect oxidation reactions, light absorbed by a photo sensitizer can react with molecular oxygen to form the more reactive singlet oxygen species.

4. THERMAL/HUMIDITY

Solid state stability can be evaluated utilizing accelerated storage temperatures in general greater than 50°C and not more than 5% relative humidity[8]. The duration of exposure is dependent on the API sensitivity. If the forced degradation thermal/humidity conditions produce a phase change, it is recommended to also run thermal/humidity conditions below the critical thermal/humidity that produces the phase change. Arrhenius kinetics may be used to establish an appropriate temperature and maximum duration of thermal degradation studies. Deviation from Arrhenius kinetics[9] is increasingly expected above 70-80°C, and the impact of this should be considered during experimental design.Using an appropriate assumption of activation energy, the duration of controlled room temperatures storage that is simulated by the study can be estimated. In general, an activation energy assumption of 15kcal/mol is recommended.

2.1OBJECTIVE:

- To develop an accurate, precise, linear, robust, simple and rapidstability indicating HPLC method for the determination of linagliptin.
- Validation of the proposed newly developed method in accordance with the analytical parameters mentioned in the ICH guidelines.
- To apply the newly developed method for analysis of the linagliptin in their bulk and pharmaceutical dosage form.
- To perform stress degradation studies for linagliptin.

2.2 SCOPE:

This study makes an attempt to establish sensitive and accurate method for estimation of anti-diabetic drug linagliptinin pharmaceutical dosage form.To date there is no study related to stability indicating HPLC method for determination of linagliptin reported in literature up to our knowledge. Hence there is strong need to develop stability indicating HPLC method for determination of linagliptin. By performing this study, reactivechemistry of linagliptin will be known. It is useful to anticipate future stability issues of both drug substance and drug product by performing forced degradation studies in acid, alkali, heat and oxidation. This study provides useful information for formulation and stability.

2.3PLAN OF WORK:

1. Carry out preliminary studies of linagliptin such as

    a. Determination of melting point

b. Determination of λ_{max} etc.,

2. Conducting initial chromatographic studies

3. Optimization of chromatographic conditions like

    a. Optimization of mobile phase

    b. Optimization of column

    c. Optimization of detection method.

d. Optimization of flow rate etc.,

4. Estimating validation parameters for optimized methods according to guidelines stated inICH.

Typical validation parameters are

a. Accuracy
b. Precision
c. Specificity
d. Linearity
e. Range
f. LOD
g. LOQ
h. Robustness

5. Performing the induced degradation studies under different stress conditions

    a. Acidic hydrolysis

    b. Basic hydrolysis

    c. Thermal degradation

    d. Oxidative degradation etc.,

2.4LITERATURE REVIEW

1. Lakshmi B et al., reported on linagliptin determination by simple, precise and accurate RP-HPLC method and validated for assay of linagliptin in tablet dosage form. Isocratic elution at a flow rate of 1.0 mL/min was employed on a symmetry Chromosil C18 column (250x4.6mm, 5µm) at ambient temperature. The mobile phase consisted of acetonitrile: water: methanol (25:50:25), detection wavelength was 238 nm and 20µL sample was injected. The retention time for linagliptin was 7 min and method was validated as per the ICH guidelines [(1)]

2. Archana M et al., developed a validated and novel isocratic reverse phase liquid chromatography method for determination of linagliptin. The chromatographic column conditions employed were Khromosil C18 (150×4.6 mm, 5µm), mobile phase containing 0.02 M potassium dihydrogen phosphate: acetonitrile (70:30, v/v, pH 5.0 adjusted with 1% OPA solution) flow rate was 1.2 mL/min and effluents were monitored at 226 nm.[(4)]

3.Ramzia I, et al.,developed a reversed-phase liquid chromatographic method for the determination of linagliptin based on isocratic elution using a mobile phase consisting of potassium dihydrogen phosphate buffer pH 4.6 - acetonitrile (20:80) at a flow rate of 1 mL/min, with cyanide symmetry column (150 x 4.6 mm, 5µm). Two detection techniques have been applied either UV detection at 299 nm in the first method or fluorometric detection at 239 nm for excitation and 355 nm for emission in the second method. Linearity, accuracy and precision were found to be acceptable over the concentration ranges of 2.5-80 µg/mL for linagliptin in bulk and 2.5-15 µg/mL for linagliptin in plasma with the first method and 5-160 µg/mL for linagliptin in bulk with the second method.[(3)]

4. LakshmanRaju B, et al., developed a HPLC method for the determination of linagliptin in pharmaceutical formulations, including the separation of impurities and excipients has been developed and validated. Isocratic elution at a flow rate of 1mL/ min was employed on a symmetry C18column at ambient temperature. The mobile phase

consisted of methanol: water 83:17(v/v) and pH of the mobile phase was adjusted to 4.1 with 0.1% orthophosphoricAcid . The UV detection wavelength was at 241nm, linearity was observed in concentration range of 5-30 µg/mL and retention time for linagliptin was 5.85min and method was validated as per the ICH guidelines.[5]

5.DilipPatil A et al.,developed a rapid, sensitive and economical high performance liquid chromatographic method for determination of linagliptin. The chromatography system containing reverse phase C18 column (250×4.6mm, 5µm) with a mixture of methanol: water in a ratio of 40:60 and flow rate of 1.0 mL/min. Orthophosphoric Acid was used as buffer to maintain the pH 3 of mobile phase. UV detector was used at 238 nm and 20µL sample was injected. The method was validated as per ICH guidelines. The retention time of linagliptin was found to be 7.3 min, calibration curve is linear in the range of 2-10 µg/mL. This study proved that the method can be successfully used for routine analysis of linagliptin form tablets formulation[6].

6.Gallwitzt B et al., reported on 2-year efficacy and safety of linagliptin compared with glimepiride in patients with type 2 diabetes inadequately controlled on metformin: a randomised, double-blind, non-inferiority trial. This study includes comparsion of a dipeptidyl peptidase-4 inhibitor (linagliptin) against a commonly used sulphonylurea (glimepiride). This study utilizes parallel-group, non-inferiority double-blind trial, conducted on outpatients with type 2 diabetes and glycatedhaemoglobinA(1c) (HbA(1c)) [37]6·5-10·0%. Glimipride and metformin combination was given initially and then linagliptin and metformin was given. Then (HbA(1c)) levels decreased with metformin and linagliptin. The findings could improve decision making for clinical treatment when metformin alone is insufficient.[10]

7. Thomas H,reported on initial combination with linagliptin and metformin in newly diagnosed type 2 diabetes and severe hyperglycemia. This phase 3 study, open-label treatment of severely hyperglycemic patients (HbA1c ≥11.0%) with linagliptin plus metformin resulted in a mean change in HbA1c of −3.7% ± 1.7%. Combination therapy was generally well tolerated with most adverse events being of mild or moderate

intensity. Asymptomatic hypoglycemia was reported by just 1 of 66 (1.5%) patients. These findings provide evidence in support of linagliptin plus metformin as a well-tolerated and effective treatment alternative to insulin for new-onset patients with type 2 diabetic mellitus (T2DM) and severe hyperglycemia.

8. Kavitha. K. Y, developed a simple, RP-HPLC method was established for determining linagliptin and metformin in pharmaceuticalformulations. Linagliptin and metformin degradation products were separated using C8 column withacetonitrile: water: methanol (25:50:25 (v/v/v), pH of the mobile phase was adjusted to 4.1 with 0.1% orthophosphoric acid.Detection was performed at 243 nm using a diode array detector. The method was validated using ICH guidelinesand was linear in the range 5-30µg/mL and 10-100 µg /mL for linagliptin and metformin respectivily. Good separationof both the analytes and their degradation products was achieved using this method.[12].

9.Christian F et al., conducted a study to investigate the pharmacokinetics of empagliflozin and linagliptin after co-administration to healthy volunteers. In this study sixteen healthy male subjects received treatment A (empagliflozin 50 mg once daily for 5 days), treatment B (empagliflozin 50 mg once daily and linagliptin 5 mg once daily for 7 days), treatment C (linagliptin 5 mg once daily for 7 days) sequence AB then C, or sequence C then AB. No adverse events were reported during the co-administration period and no hypoglycemia was reported. Empagliflozin and linagliptin were well tolerated. These data support the co-administration of empagliflozin and linagliptin without dose adjustments.

10. Ranjit S et al., reported current trends in forced degradation study for pharmaceutical product development. This study explains that forced degradation is a powerful tool used routinely in pharmaceutical development in order to develop stability indicating methods that lead to quality stability data and to understand the degradation pathways of the drug substances and drug products. Study describes the mechanism of formation and characterization of generated impurities during force degradation studies in

pharmaceuticals. Force degradation studies ensure appropriate stability of final pharmaceutical product in very early stages of pharmaceutical development.[31]

11. George N et al., reported on force degradation studies, explained that HPLC method should be able to separate, detect and quantify the various drug related degradants that can form on storage or manufacturing and quantify any drug related impurities that may be introduce during synthesis. Further, described the general degradation procedure in different conditions like photolytic, thermal, acidic, basic and oxidative conditions. In general, a target of approximately 10% degradation of API during force degradation or exposure to energy in slight excess of what is typically used in accelerated storage is recommended.

2.5 DRUG PROFILE

DRUG NAME: Linagliptin

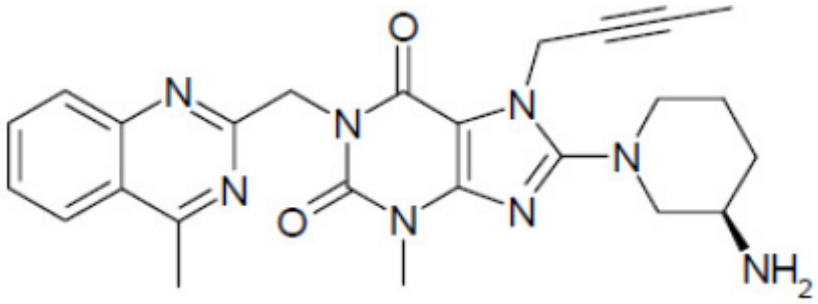
IUPAC NAME: 8-[(3*R*)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3- methyl-1-[(4-methylquinazolin-2-yl)methyl]-3,7-dihydro-1*H*-purine-2,6-dione.

CHEMICAL FORMULA: $C_{25}H_{28}N_8O_2$

MOLECULAR WEIGHT: 472.54g/mol

TRADE NAMES: Tradjenta, Trajenta

STRUCURE:



PHYSICAL STATE: Yellowish white amorphous powder

NATURE: Slightly hygroscopic

pKa : pKa_1= 8.6 ; pKa_2 = 1.9

PARTITION COEFFICIENT: logP = 1.7 (free base)

logD (pH 7.4) = 0.4

SOLUBILITY:

- Slightly soluble in water.
- Soluble in methanol.
- Sparingly soluble in ethanol.
- Very slightly soluble in isoproponol.
- Very slightly soluble in acetone.

BIOAVAILABILITY: 30%.

PROTEIN BINDING: Plasma protein binding of linagliptin is concentration-dependent, decreasing from about 99% at 1 nmol/L to 75%-89% at ≥30 nmol/L, reflecting saturation of binding to dipeptidyl peptidase-4 with increasing concentration of linagliptin. At highconcentrations, where dipeptidyl peptidase-4 is fully saturated, 70% to 80% of linagliptin remains bound to plasma proteins and 20% to 30% is unbound in plasma. Plasma binding is not altered in patients withrenal or hepatic impairment.

METABOLISM: In oral administration, the majority (about 90%) of linagliptin is excreted unchanged, indicating that metabolism represents a minor elimination pathway. Asmall fraction of absorbed linagliptinis metabolized to a pharmacologically inactive metabolite, which shows a steady-state exposure of 13.3% relative to linagliptin.

EXCRETION: In oral [14^{C}]-linagliptin dose to healthy subjects, approximately 85% of the administered radioactivity was eliminated via the enterohepatic system (80%) or urine (5%) within 4 days of dosing. Renal clearance at steady state was approximately 70 mL/min.

MECHANISM OF ACTION: Linagliptin is a dipeptidyl peptidase-4 inhibitor developed by BoehringerIngelheim (German Pharmaceutical Company) for treatment of type II diabetes[(7)]. It was approved by the US FDA on 2 May 2011 for treatment of type II diabetes**.**. DPP-4(dipeptidyl peptidase 4) is an enzyme that degrades the incretion hormones, glucagon like peptide-1 (GLP-1) and glucose- dependent insulinotropic polypeptide (GIP). Both GLP-1 and GIP increase insulin biosynthesis and secretion from pancreatic beta cells in the presence of normal and elevated blood glucose levels. GLP-1 also reduces glucagon secretion from pancreatic alpha cells, resulting in a reduction in hepatic glucose output. Thus, linagliptin stimulates the release of insulin in a glucose-dependent manner and decreases the levels of glucagon in the circulation, and showed that the drug can effectively reduce blood sugar.

3.1. MATERIALS AND METHODS

Linagliptinwas a generous gift sample from Crystal marphix Pvt Ltd, Hyderabad. Formulation product Trajenta tablets (labeled as 5mg of linagliptin per tablet) were supplied by Boehringer Ingelheim India. HPLC grade methanol and acetonitrile, analytical grade potassium dihydrogen phosphate, orthophosphoric acid and triethylamine, laboratory reagent grade hydrochloric acid, sodium hydroxide and hydrogen peroxide were purchased from Merck, India. Water was purified through Direct-Q 3 UV water purification system (Merck Millipore, India)

INSTRUMENTS:

Name of the instrument	Make
Sonicator	Analytical Technologied ltd
Cyclo mixer	Remi
Weighing balance	Sartorious balance
HPLC instrument (manual)	Analytical Technologies ltd P2230 with A-2000 software and version 1.6.
HPLC column: MicrosorbC-8 column (150 mm x 4.6 mm, 5 µm)	Ranin instrument company, USA
pH meter	Systronics

3.1.1. PRELIMINARY STUDY

3.1.2. DETERMINATION OF MAXIMUM WAVELENGTH (λ_{max})OF ABSORPTION OF LINAGLIPTIN:

Maximum wavelength (λ_{max})of linagliptin[38] was established by scanning the drug solution in UV. From the stock solution (1mg/mL)200µl of sample is diluted to 10ml with methanol to obtain 20µg/ml solution and scanned in UV region.

3.1.3. DETERMINATION OF LINEARITY IN UV:

Linearity was established by least squares linear regression analysis of the calibration curve. The constructed calibration curve was linear over concentration range of 2 µg/ml to 25µg/ml at 295nm and 0.8 µg/ml to 8 µg/ml at 225nm.

3.1.4. HPLC INSTRUMENTATION AND CONDITIONS:

The HPLC system consisted of Analytical technology model 2230 equipped with UV detector and data collection as well as analysis were carried out through A2000 software. The elution was performed with following conditions

Flow rate : 1.0 mL/min

Column : MicrosorbC8 column (150 mm x 4.6 mm, 5 µm)

Mobile phase : 40 mM phosphate buffer (pH 3), and acetonitrile (70:30,v/v)

Detector wavelength : 225 nm

Column temperature : Ambient temperature

Injection volume : 20 µL

Run time : 10 min

3.1.5. BUFFER PREPARATION:

5.4436gm of potassium dihydrogen phosphate was dissolved in HPLC water and transferred to 1000 mL volumetric flask, volume was made up to 1000ml with HPLC water, pH of the solution adjusted to 3 with ortho phosphoric acid. Then buffer was filtered through 0.45 µm filter.

3.1.6. PREPARATION OF STANDARD STOCK SOLUTION

Standard stock solution of linagliptin (1000µg/mL) was prepared in methanol by dissolving 50mg of linagliptin in 50 mL of methanol. The required concentrations prepared by serial dilutions of the stock solution.

3.1.7.SAMPLE PREPARATION

Ten tablets were weighed, their mean weight was determined, and crushed in a mortar. An amount of powdered mass equivalent to one tablet content was transferred into a 50mL volumetric flask.To it add 10mL of methanol and mechanically shaken for 10min, ultra sonicated for 5min and make up to the volume up to 50mL with methanol. From this 1mL is taken then diluted with mobile phase up to 10mL, filtered through 0.2µm nylon filter.

3.1.8. METHOD VALIDATION[33]

3.1.8.1. SYSTEM SUITABILITY

System suitability was determined from six replicate injections of standard preparation (10 µg/mL), which was prepared from appropriately diluting standard stock solution. All important characteristics including retention time, tailing factor, capacity factor, peak resolution, and theoretical plate number were analyzed.

Acceptance criteria

1. The % RSD (relative standard deviation) for the retention times of linagliptin peak from 6 replicate injections of each standard solution should be not more than 2.0%.
2. The number of theoretical plates (N) for linagliptin peaks should be not less than 2000.
3. The tailing factor (T) for linagliptin the peaks should be not more than 2.0.

3.1.9. LINEARITY

Accurately measured aliquots of standard stock solution contacting 2-160 µg of linaglptin were transferred into 10 mL volumetric flasks in triplicate and volume was made up to 10 mL with mobile phase. Each solution was analyzed by injecting 20µL volume. The calibration curve was obtained by plotting peak area against concentration. Further the linearity[34] was evaluated by the determination of standard deviation (SD), slope, intercept and correlation coefficient (r^2).

Acceptance criteria

1. Correlation coefficient should be not less than 0.999.
2. % RSD of peak areas for solutions should not be more than 2.0%.

3.1.10. LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

Limit of detection (LOD) and Limit of quantification (LOQ) were determined based on the standard deviation of the response and the slope. The standard deviation of the response and the slope of the calibration curve were calculated from the slope and y-intercept of the mean equation of three calibration curves[43].

The limit of detection was calculated by using the formula

$$LOD = \frac{3.3\ \sigma}{S}$$

where

σ = the standard deviation of the response

S = the slope of the calibration curve

The limit of quantification was calculated by using the formula

$$LOQ = \frac{10\ \sigma}{S}$$

where

σ = the standard deviation of the response

S = the slope of the calibration curve.

3.1.11. SPECIFICITY

The specificity[36] of the HPLC method was determined by the complete separation of linagliptin along with other parameters like retention time (RT), capacity factor (k), tailing or asymmetrical factor (T), etc.

Acceptance criteria

1. Chromatogram of standard and sample should be identical with near retention time.
2. Chromatogram of blank should not show any peak at the retention time of analyte peak.

There is no interference due to blank at the retention time of analyte. Hence the method is specific.

3.1.12. PRECISION

The repeatability of the proposed HPLC method was assessed by three replicate injection of three different concentrations (6, 10 and 14 µg/mL) of linagliptin, representing 60, 100, 140%, respectively[42]. The experiments described in repeatability were repeated for three consecutive days to establish intermediate precision.

Acceptance criteria

1. % RSD of peak areas should not be more than 2.0%.

2.1.13. ACCURACY

To establish accuracy, recovery studies were carried out by spiking known standard samples into sample solution. Known amount of the standards at 80,100 and 120% levels were fortified to sample solution. Peak areas of standards were calculated by the difference of peak areas between fortified and unfortified sample[22]. Three replicate samples of each concentration level were prepared and the recovery at each level (n = 3), and mean recovery (n = 9) were determined. The percentage recoveries were calculated from the slope and y-intercept of the calibration curve[39].

Acceptance criteria

1. The mean % recovery of linagliptin at each spike level should be not less than 98.0% and not more than 102.0%.

3.1.14. ROBUSTNESS

The robustness of the method was evaluated through the studies of influence of small and premeditated alteration of analytical parameters[23]. The parameters selected were flow rate (±10%) mobile phase composition (acetonitrile ±3%), pH of the buffer (±7%) and buffer concentration (±12%). Only one parameter was altered while the remaining parameters were kept constant.

Effect of variation flow rate

A study was conducted to determine the effect of variation in flow rate[40]. Standard solution was prepared and injected in to the HPLC system by keeping flow rates 0.9mL/min and 1.1mL/min. The effect of variation of flow rate was evaluated.

Acceptance criteria

1. The %RSD of tailing factor and retention times of linagliptin standard should not be more than 2.0.

Effect of variation of mobile phase composition

A study was conducted to determine the effect of variation in mobile phase ratio by changing the ratio of mobile phase[41] i.e., acetonitrile ±3% (v/v), standard solution was prepared and injected in to the HPLC system. The effect of variation of mobile phase was evaluated.

Acceptance criteria

2. The %RSD of tailing factor and retention times of linagliptin standard should not be more than 2.0 variations in composition of mobile phase.

Effect of variation of pH

A study was conducted to determine the effect of variation in pH by changing the pH of buffer[45]i.e. ±7%. Standard solution was prepared and injected into the HPLC system. The effect of variation of pH was evaluated.

1. The tailing factor of linagliptin standard should not be more than 2.0 for variation in pH.

2. The %RSD of asymmetry and retention time of linagliptin standard should be not more than 2.0% for variation in pH.[48]

Effect of variation in Buffer concentration

A study was conducted to determine the effect of variation in buffer concentration by changing the buffer concentration[44] i.e. ± 12% . Standard solution was prepared and injected into HPLC system. The effect of variation of buffer concentration was evaluated.

Acceptance criteria

1. The tailing factor of linagliptin standard should not be more than 2.0 for variation in buffer concentration[47].
2. The %RSD of asymmetry and retention time of linagliptin standard should be not more than 2.0% for variation in buffer concentration.

3.1.15. FORCED DEGRADATION STUDIES(32)

HYDROGEN PEROXIDE INDUCED DEGRADATION

The standard stock solution (0.1mL) of was transferred into 10 mL volumetric flask, added with 3 mL of 3% hydrogen peroxide solution The solution was kept at room temperature for 3 hours and diluted to 10mL with mobile phase. The 20 µL of the resultant solution was injected and analysed.

THERMAL DEGRADATION

The 10 mg of linagliptin API was transferred into a clean 10mL volumetric flask, placed in oven at 60˚C for 10 days to evaluate dry heat degradation. After 10 days dissolved and volume was made up with methanol. Further serial dilutions were made with mobile phase to attain 10 µg/Mlconcentration, 20 µL of the resultant solution was injected and analysed.

ACID INDUCED DEGRADATION

To 0.1 mL of linagliptin stock solution in different 10 mL volumetric flasks, 3 mL of either 0.1N or 1N hydrochloric acid was added, separately. The mixtures were kept at room temperature for 3 hr, 24 hr and 48 hr duration[49], neutralized with appropriate amount of either 0.1N or 1N sodium hydroxide solution or volume was made up to 10 mL with mobile phase. The similar experiments repeated at higher temperature of 60˚C for 3 hr duration. The 20 µL of the resultant solution was injected and analysed.

BASE INDUCED DEGRADATION

To 0.1 mL of linagliptin stock solution in different 10 mL volumetric flasks, 3 mL of either 0.1N or 1N sodium hydroxide solution was added, separately. The mixtures were kept at room temperature for 3 hr, 24 hr and 48 hr duration, neutralized with appropriate amount of either 0.1N or 1N hydrochloric acid or volume was made up to 10 mL with mobile phase. The similar experiments repeated at higher temperature of 60˚C for 3 hr duration. The 20 µL of the resultant solution was injected and analysed.[50]

4. RESULTS

4.1. PRELIMINARY STUDY

4.1.1. DETERMINATION OF MAXIMUM WAVELENGTH OF ABSORPTION(λ_{max}) OF LINAGLIPTIN:

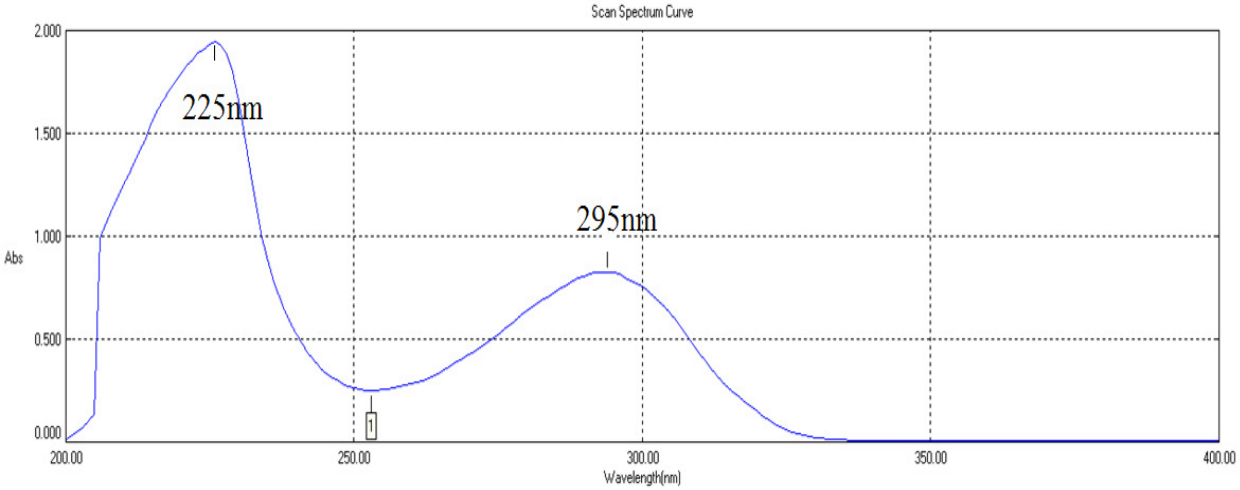


Figure: 4.1. Scan spectra of linagliptin

4.1.2. DETERMINATION OF LINEARITY IN UV

Table No 4.1. Results of calibration curve at 295nm in UV

Concentration	Absorbance at 295nm
2	0.079
5	0.193
8	0.29
10	0.421
12	0.487
15	0.676
20	0.803
25	1.054

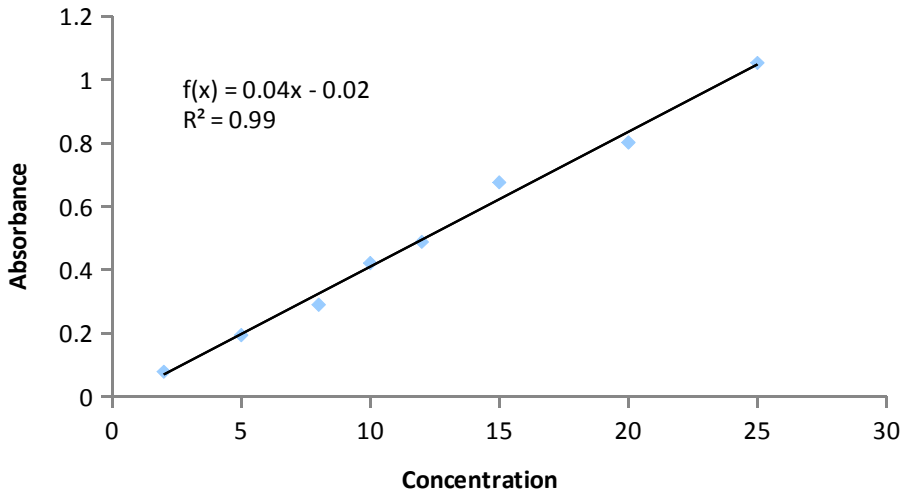


Figure:4.2. Calibration curve in UV at 295nm

Table No 4.2. Results of calibration curve at 225nm in UV

concentration	Absorbance at 225nm
0.8	0.129
1	0.146
2	0.259
3	0.428
4	0.51
5	0.658
6	0.756
7	0.921
8	0.996

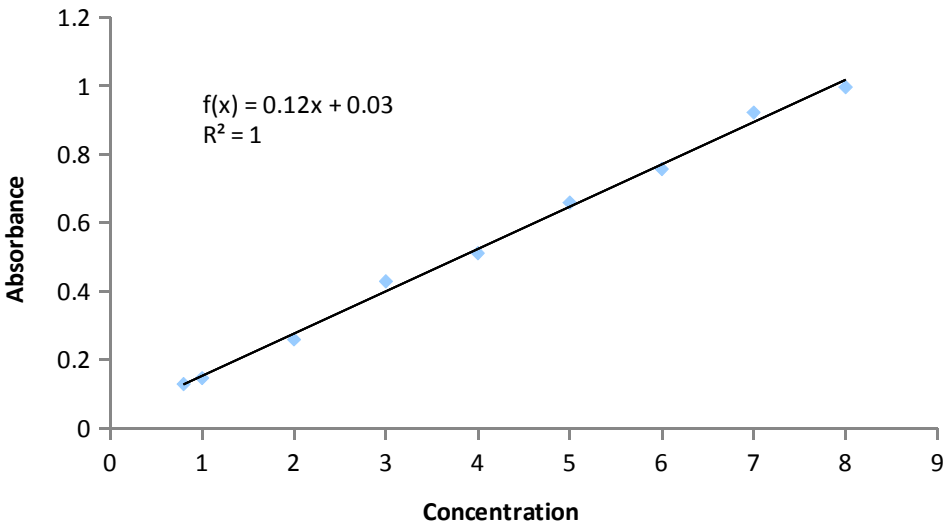


Figure: 4.3 Calibration curve in UV at 225nm

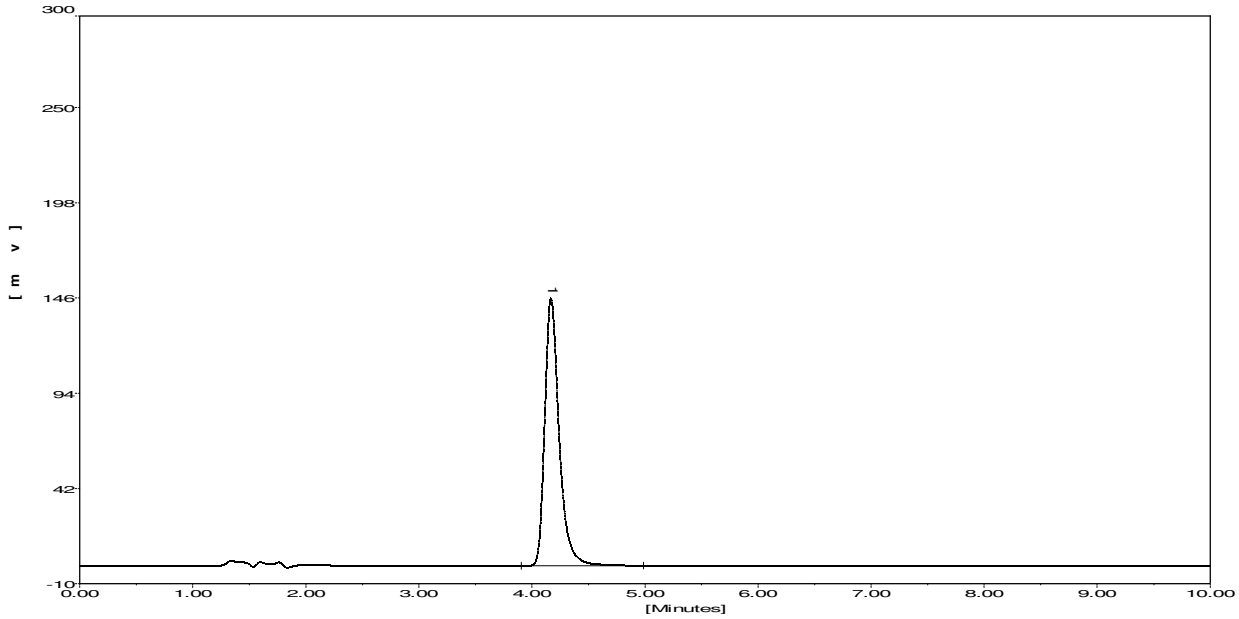


Figure: 4.4. A typical chromatogram of 20µL injection of linagliptin standard solution (10µg/mL)

Peak area	Tailing factor	Retention time	Theoretical plates
1289	1.56	4.17	23045

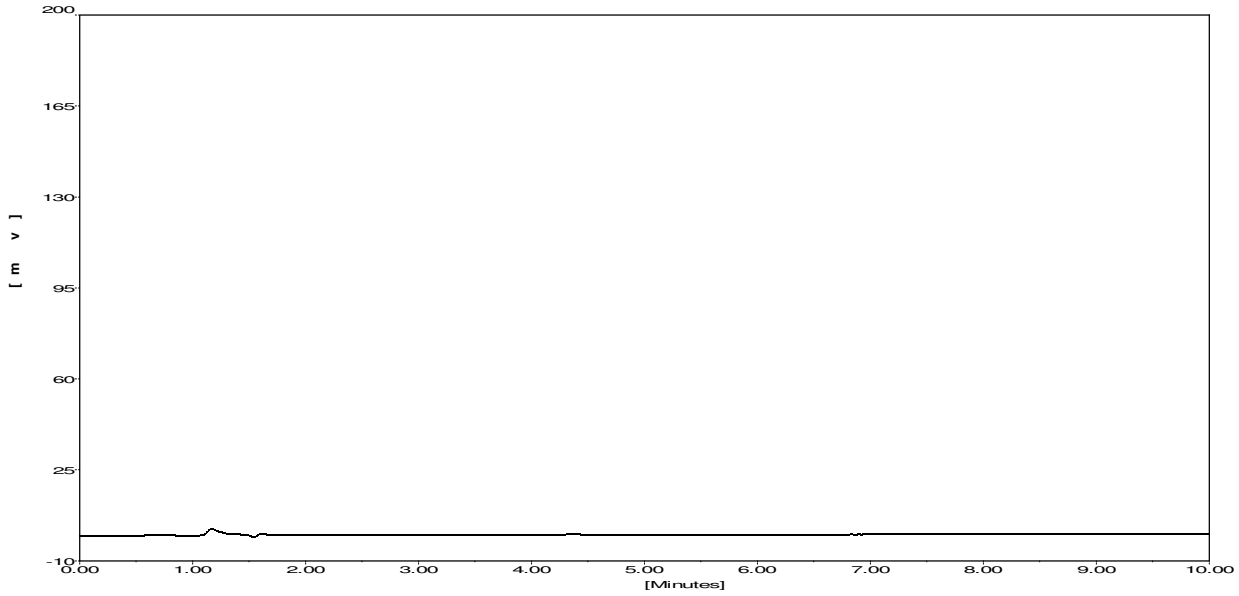


Figure: 4.5. A typical chromatogram of blank

METHOD VALIDATION

Table No 4.3. System suitability parameters

Property	Mean ± SD (n=6)	% RSD	Required limits
Retention time	4.19 ± 0.03	0.71	≤ 2%
Capacity factor	2.30 ± 0.002	0.087	2-5
Theoretical plates	22364 ± 198	0.88	> 2000
Tailing factor	1.52 ± 0.004	0.26	≤ 2

LINEARITY

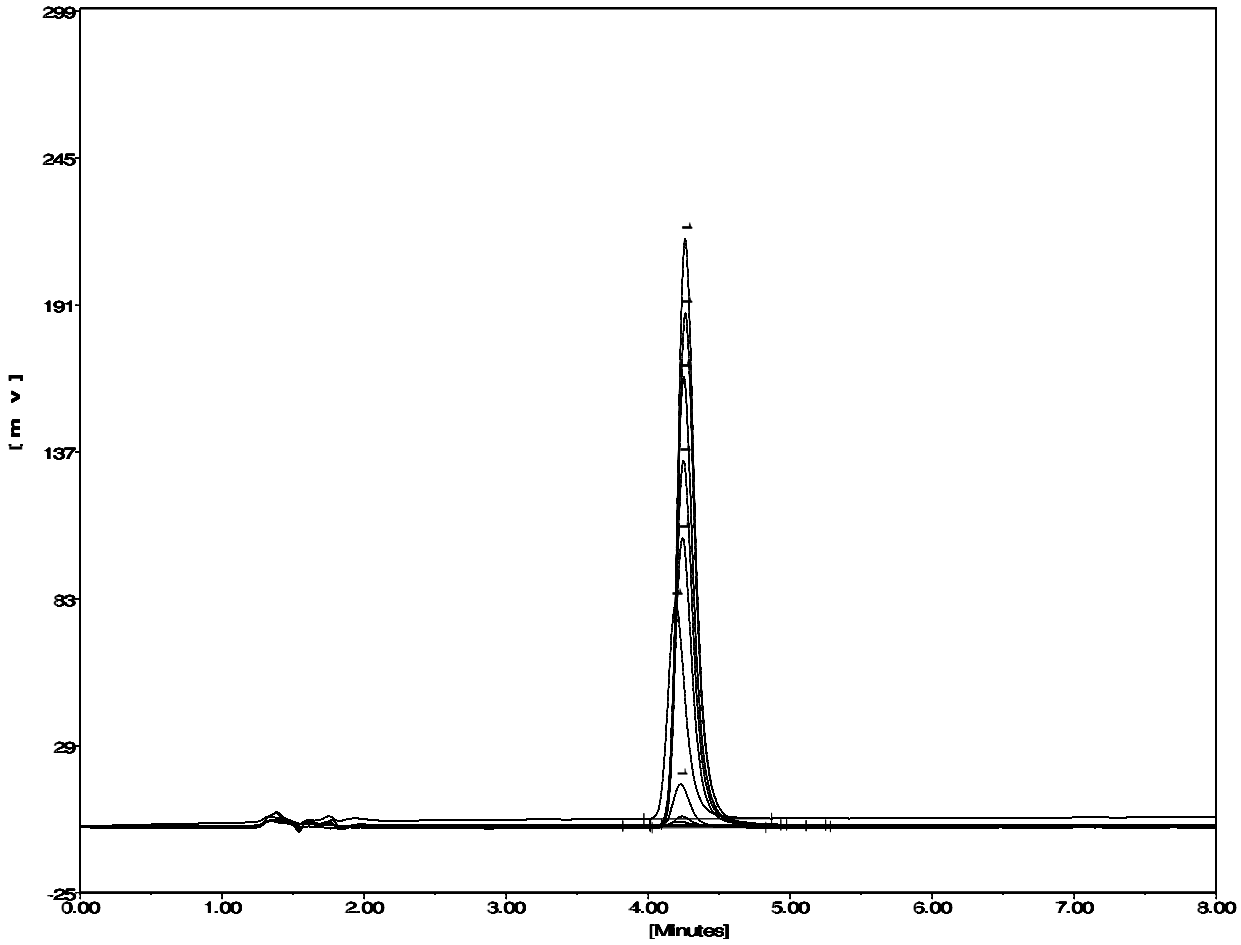


Figure: 4.6. Overlay of the chromatograms of linagliptin solutions (0.2 – 16 µg/mL concentration)

Table No: 4.4.Calibration curve data of linagliptin

Concentration (µg/mL)	Mean peak area ± SD	Tailing factor	Retention time (min)	Theoretical plates/meter
0.2	22.323± 0.288675	1.58	4.24	22279
0.5	74.466± 0.321438	1.61	4.21	21798
1	155.559± 0.404145	1.57	4.22	22834
6	791.666± 2.516611	1.59	4.19	21710
8	1031.333 ± 6.506407	1.59	4.24	22771
10	1286.667 ± 4.932883	1.62	4.25	22833
12	1520.667 ± 7.371115	1.62	4.25	22578
14	1792.333 ± 3.511885	1.65	4.26	22691
16	2014.333 ± 4.50925	1.63	4.26	23547

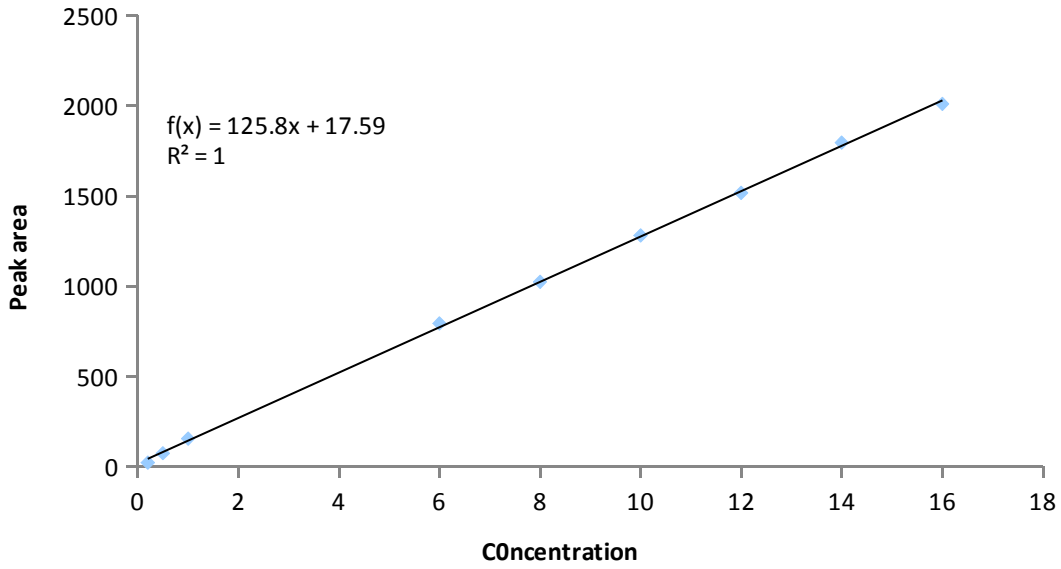


Figure: 4.7. Calibration curve of linagliptin

LOD AND LOQ

LOD = 3.3 X σ / S

σ = the standard deviation of the response = 0.3162

S = the slope of the calibration curve = 125.93

LOD = (3.3 X 0.3162) / 125.93

= 0.008µg/mL

LOQ = 10 X σ / S

σ = the standard deviation of the response = 0.3162

S = the slope of the calibration curve = 125.93

= (10 X 0.3162) / 125.93

= 0.025 µg/mL

Table No 4.5 LOD and LOQ of linagliptin

LOD(µg/mL)	LOQ(µg/mL)
0.008	0.025

ACCURACY

Table No: 4.6. Recovery studies of lingliptin spiked with formulation by standard addition method.

Concentration (%)	Volume of standard solution (100µg/mL) (mL)	Volume of spiked sample solution (100µg/mL) (mL)	Total concentration obtained	Concentration calculated, ±SD, % RSD	% recovery
80%	0.8mL	1 mL	18µg/mL	18.33±0.21, 0.73	101.83%
100%	1mL	1mL	20µg/mL	20.34±0.17, 0.42	101.52%
120%	1.2mL	1mL	22µg/mL	22.07±0.51, 1.02	100.31%

SPECIFICITY

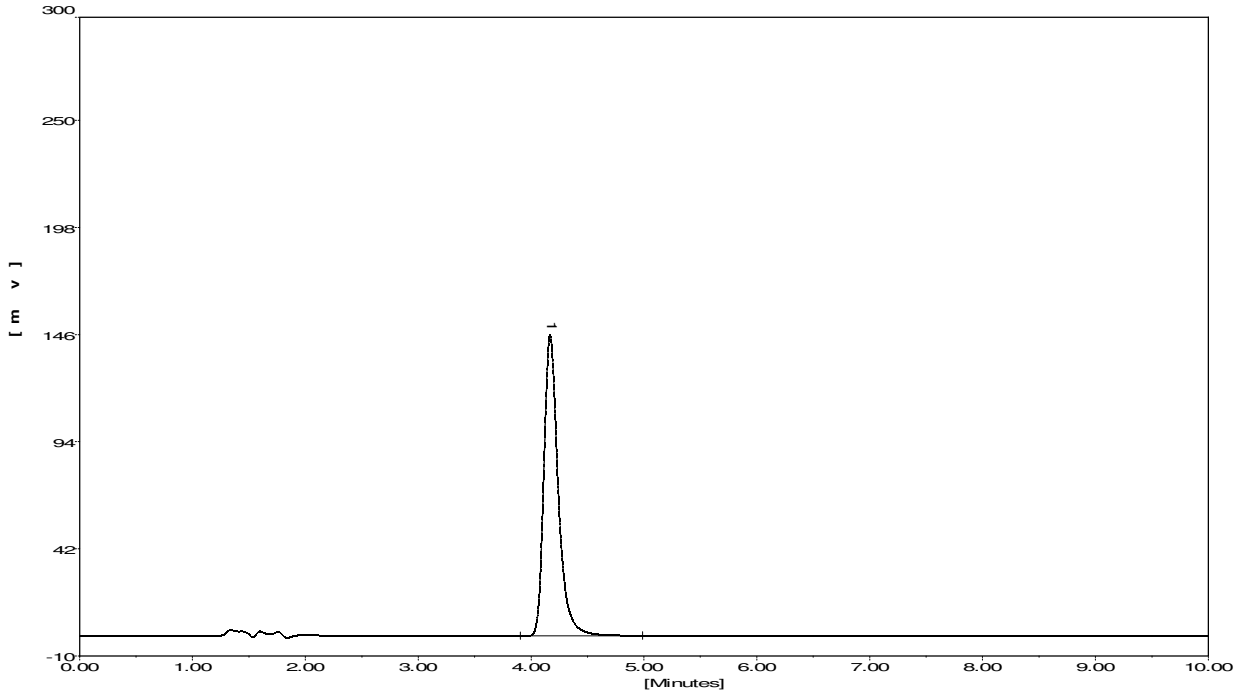


Figure: 4.8. A typical chromatogram of 20µL injection of linagliptin standard solution (10µg/mL)

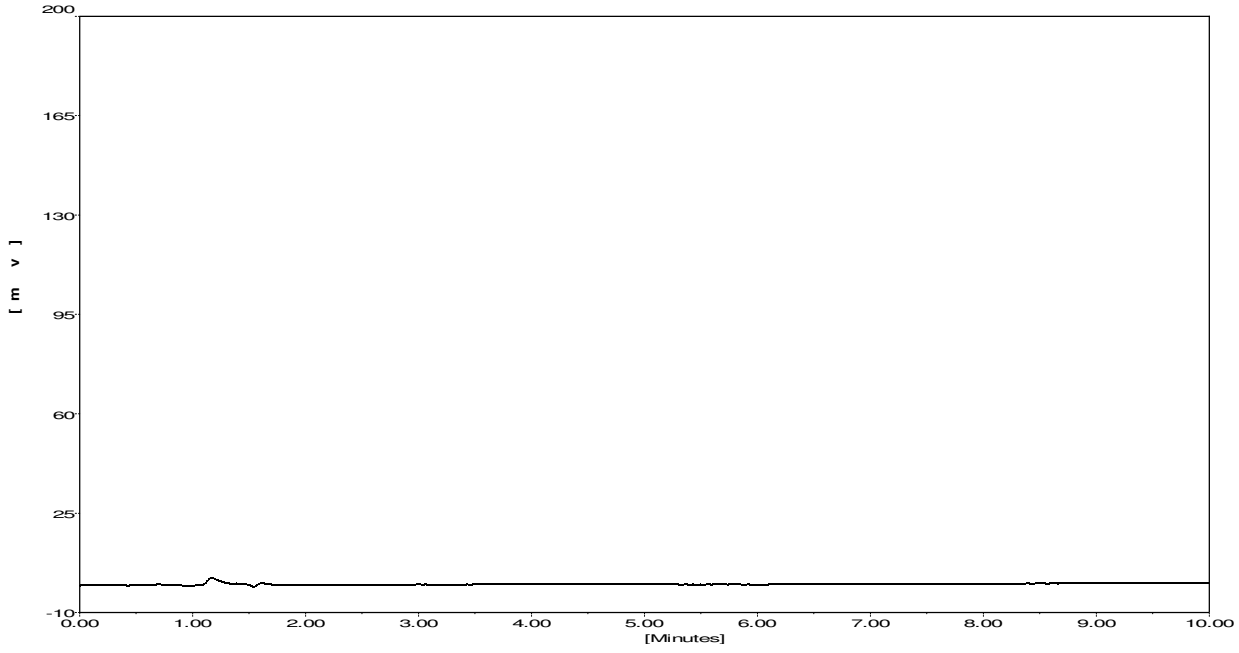


Figure: 4.9. A typical chromatogram of blank

PRECISION

SYSTEM PRECISION

Table No: 4.7. Results of system precision

Concentration (µg/ml)	Intraday precision		Inter day precision	
	Mean peak area ± SD, % RSD,(n=6)	Mean retention time ± SD, % RSD (n=6)	Mean peak area ± SD, % RSD,(n=18)	Mean retention time ± SD, % RSD (n=18)
10	1295 ± 18.81, 1.45	4.18 ± 0.0008, 0.19	1261 ± 16.69, 1.32	4.22 ± 0.03, 0.787

METHOD PRECISION

Table No 4.8. Results of method precision

Concentration (µg/ml)	Intraday precision		Inter day precision	
	Mean peak area ± SD, % RSD,(n=3)	Mean retention time ± SD, % RSD (n=3)	Mean peak area ± SD, % RSD,(n=9)	Mean retention time ± SD, % RSD (n=9)
6	796.66 ± 0.015, 1.56	4.24 ± 0.005 , 0.13	801 ± 0.133, 1.17	4.24 ± 0.0051, 0.121
10	1289 ± 0.051, 0.50	4.26 ± 0.025, 0.59	1283.11 ± 0.06677, 0.66	4.26 ± 0.022, 0.527
14	1770 ± 0.049, 0.35	4.24 ± 0.02, 0.49	1770.66± 0.067, 0.48	4.24 ± 0.0150, 0.354

ASSAY OF LINALIPTIN IN MARKETED FORMULATION

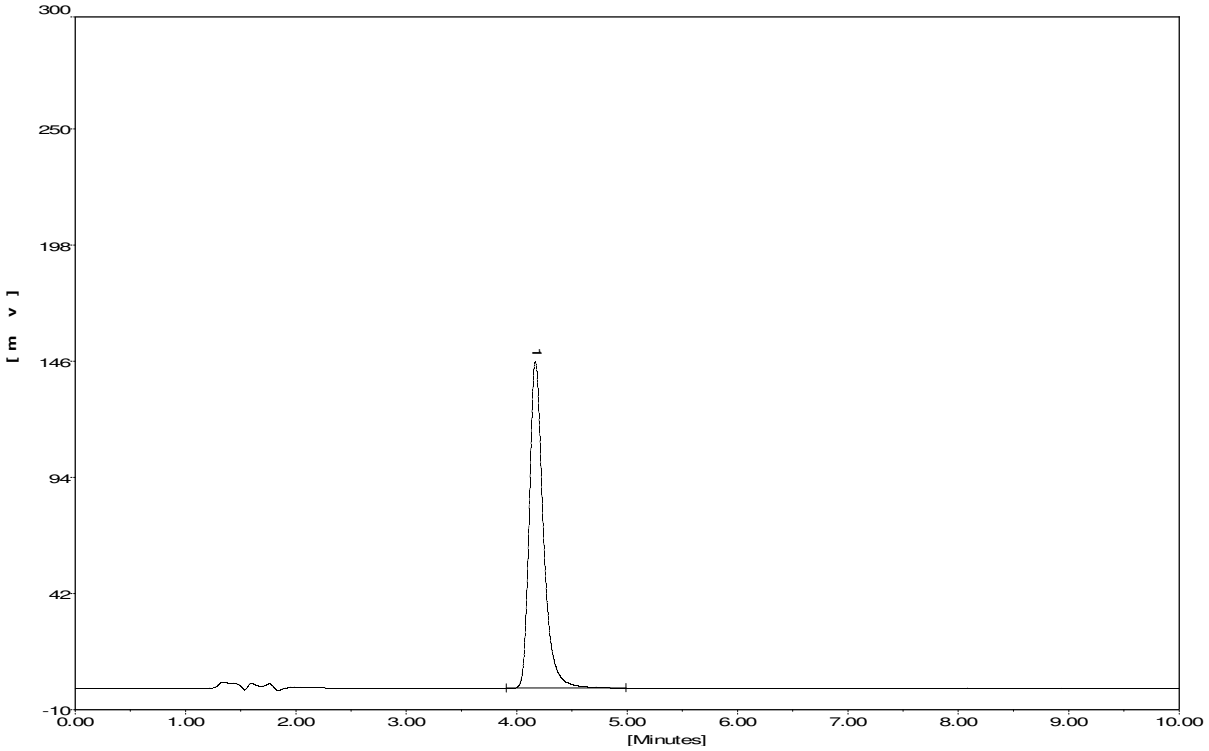


Figure: 4.10. Chromatogram of assay of linagliptin in marketed formulation Trajenta

Table No 4.9. Results of assay of linagliptin

Brand name	Labeled amount (mg)	Calculated concentration (mg) ± SD (N = 3)	Assay %
Trajenta	5	5.05	101%

$$y = 125.8x + 17.58$$

ROBUSTNESS

Table No: 4.10. Results of robustness

VARIABLE FACTOR	LEVEL	RETENTION TIME	TAILING FACTOR	PEAK AREA
Change in flow rate	Decreased flow rate (0.9mL/min)	4.76	1.40	1234
	Nominal flow rate (1mL/min)	4.49	1.42	1261
	Increased flow rate(1.1mL/min)	4.18	1.42	1195
Change in ACN composition	DecreasedACN concentration (29%)	4.99	1.41	1199
	Nominal ACN concentration(30%)	4.49	1.42	1260
	Increased ACN concentration(31%)	4.08	1.42	1206
Change in buffer pH	Decreased buffer (pH 2.8)	4.43	1.42	1210
	Nominal buffer (pH 3)	4.49	1.42	1260
	Increasedbuffer (pH 3.2)	4.54	1.42	1221
Change in buffer concentration	Decreasedbuffer concentration (35mM)	4.51	1.43	1243
	Nominal buffer concentration(40mM)	4.49	1.42	1261
	Increased buffer concentration(45mM)	4.41	1.41	1224

SOLUTION STABILITY

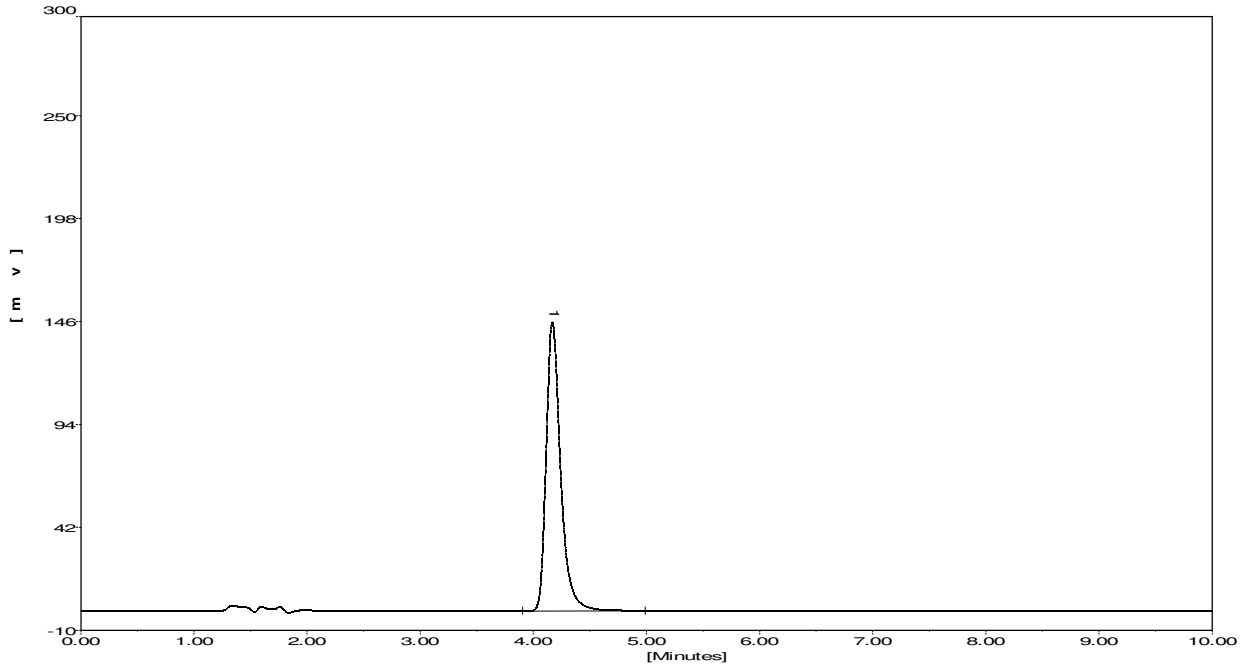
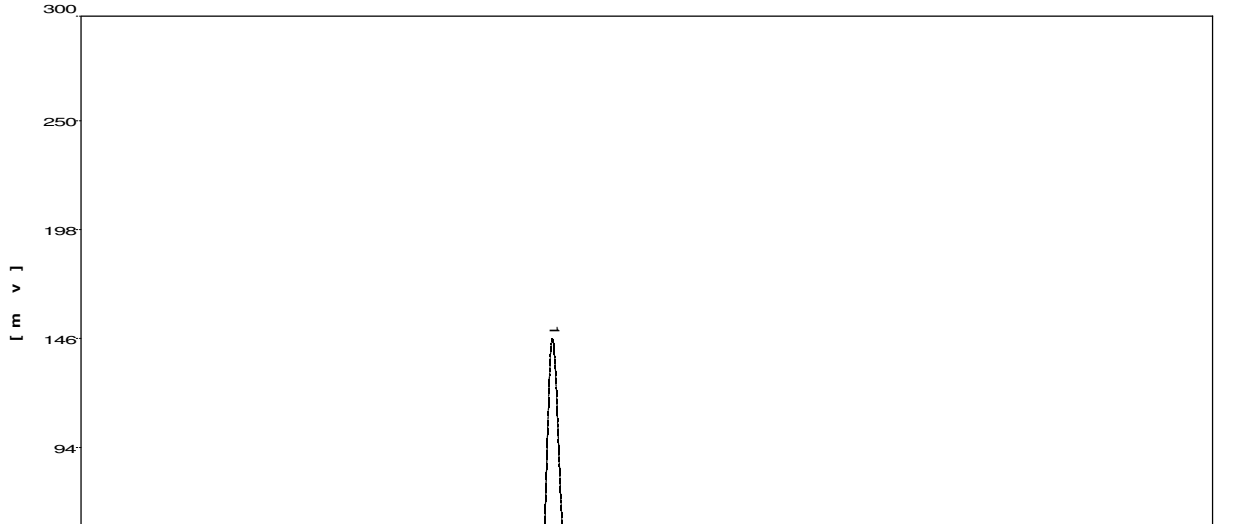


Figure: 4.11. Chromatogram indicating solution stability of linagliptin (1hr)



Time	Peak area
0	1284
15min	1297
30min	1262
1hr	1291
2hr	1282
3hr	1290
24hr	1289
Mean ± SD	1284.33 ± 12.17
% RSD	0.98

FORCED DEGRADATION STUDIES

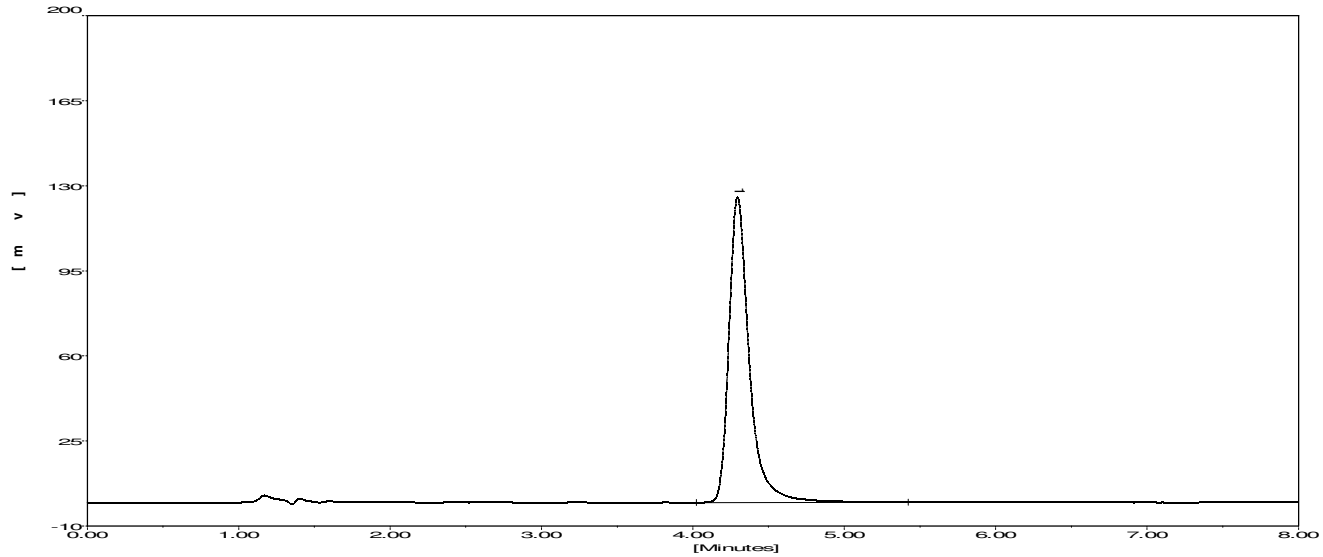


Figure: 4.13.Standard chromatogram of linagliptin (10µg/mL) for comparing degradation samples

Peak area	Tailing factor	Retention time	Theoretical plates

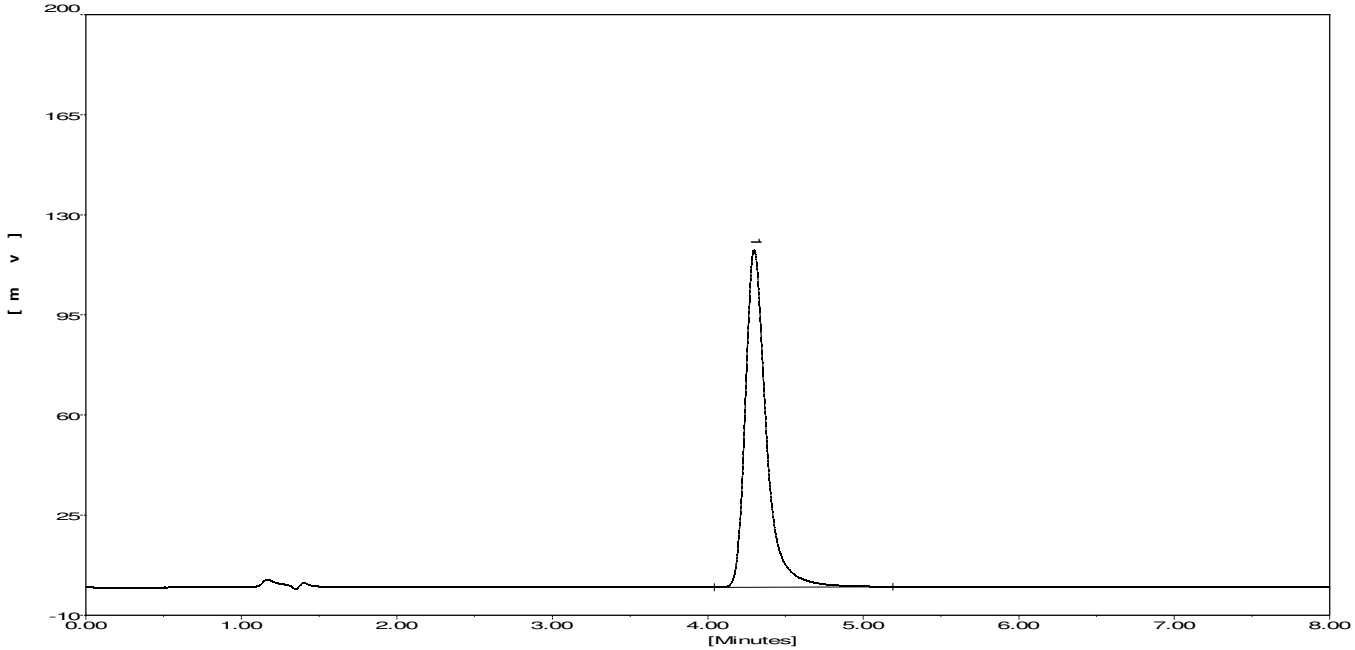
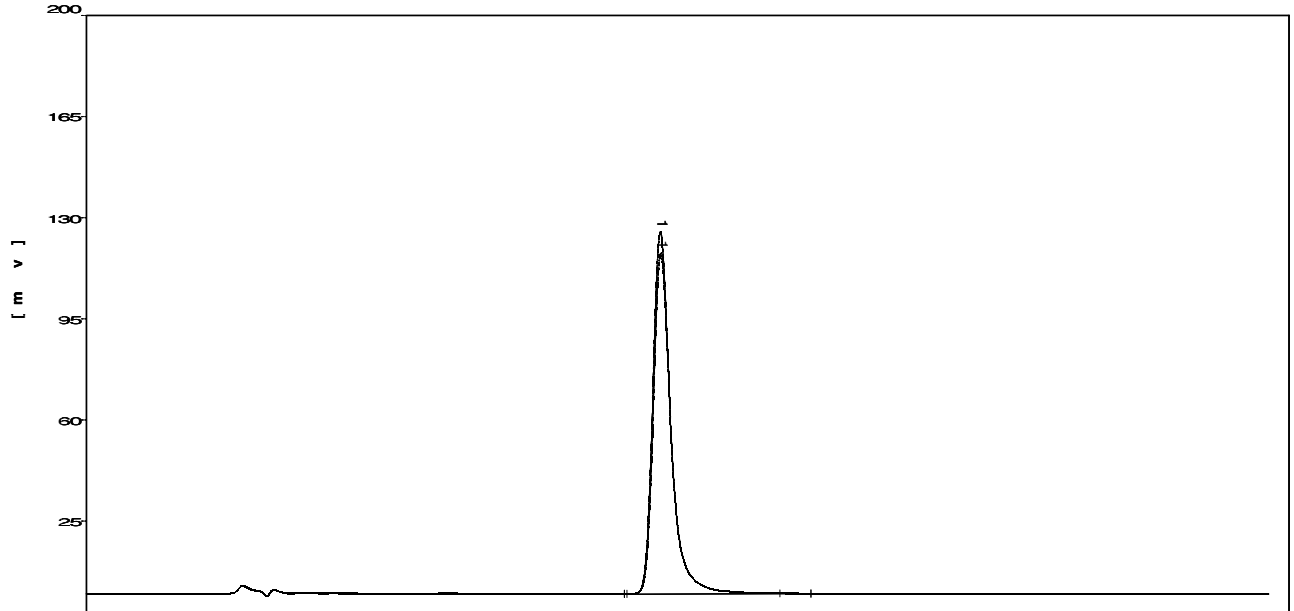


Figure: 4.14 Chromatogram of dry heat degraded linagliptin



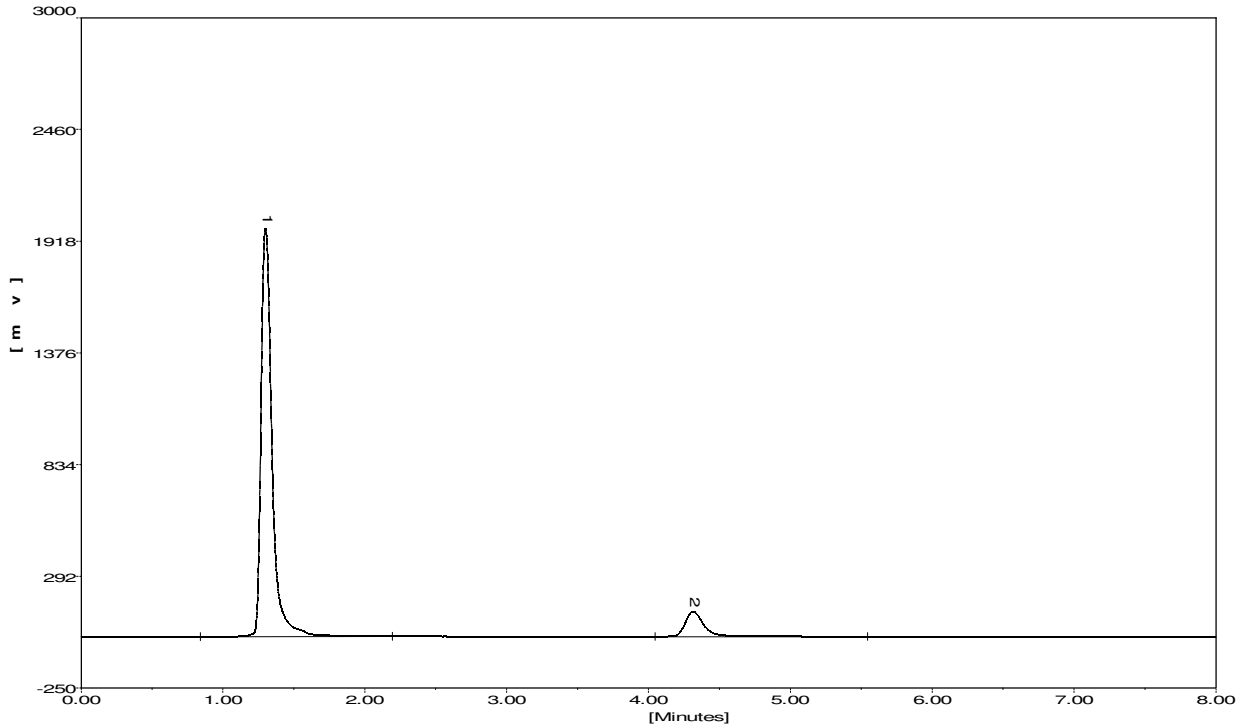


Figure: 4.16Chromatogram of linagliptin degraded with 3% H_2O_2

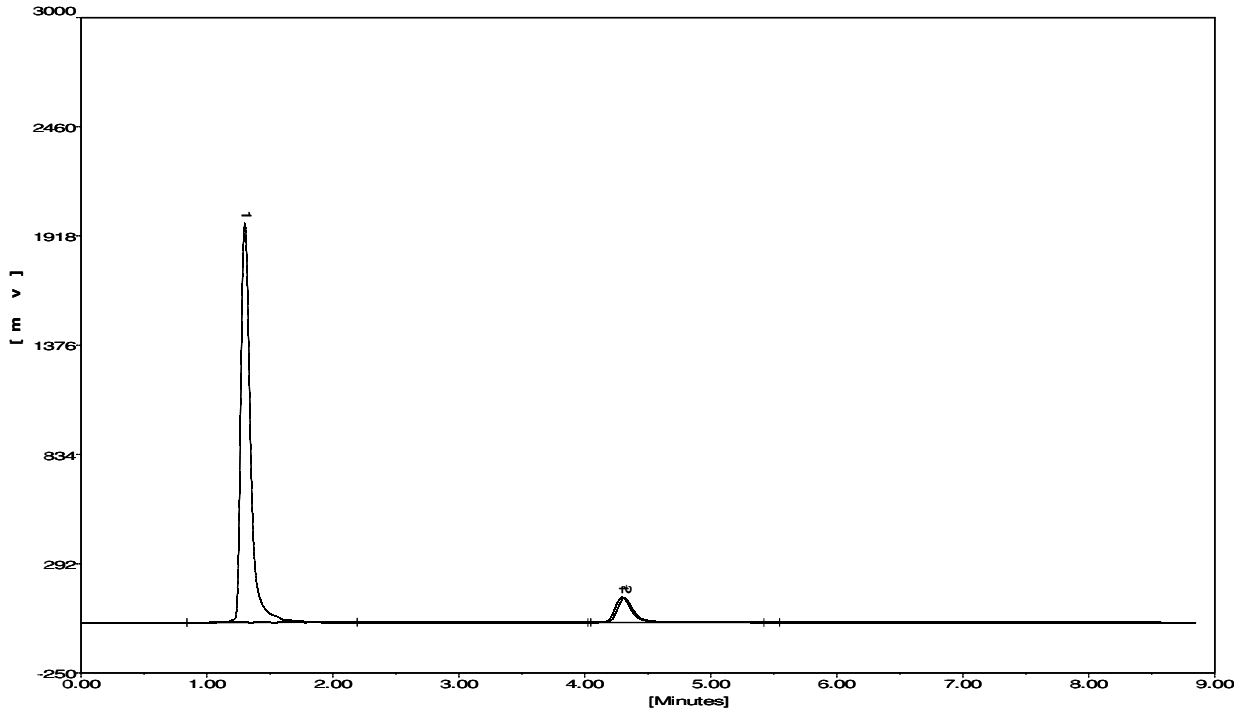
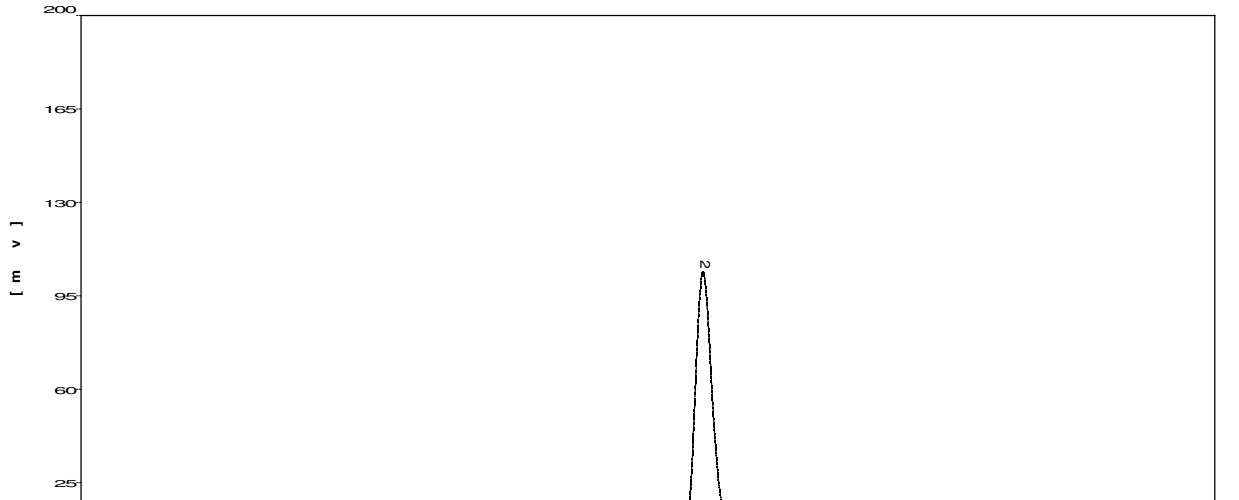


Figure: 4.17. Overlapping chromatograms in 3% H_2O_2 and standard linagliptin

ACID INDUCED DEGRADATION



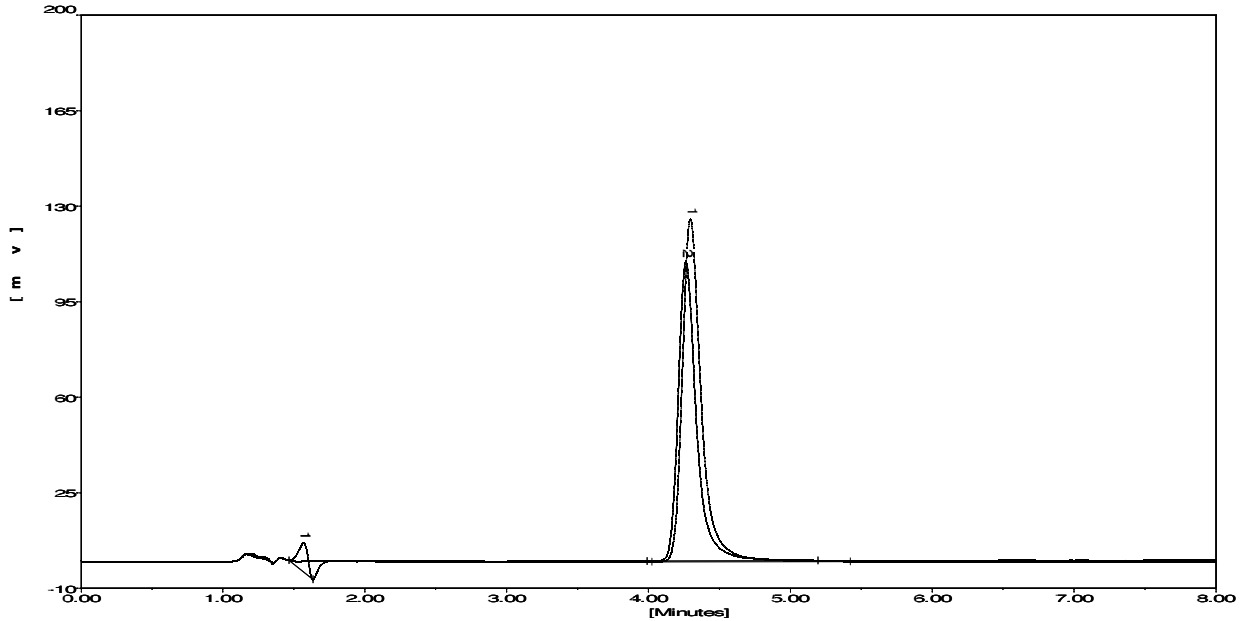
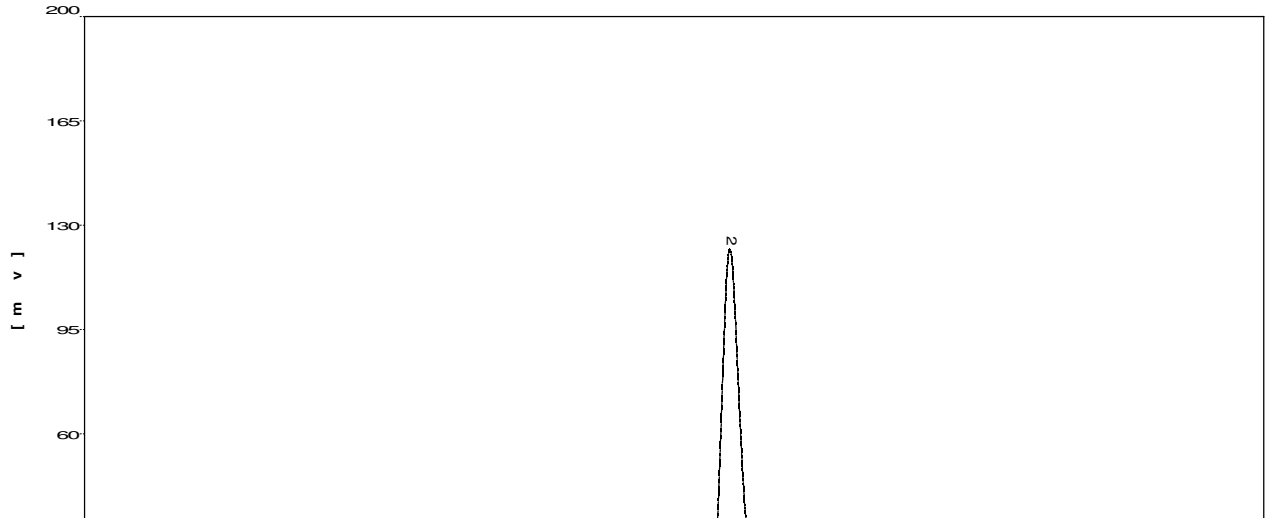


Figure: 4.19. Overlapping chromatograms of linagliptin treated with 0.1N HCl at room temperature for 3 hours and standard linagliptin.



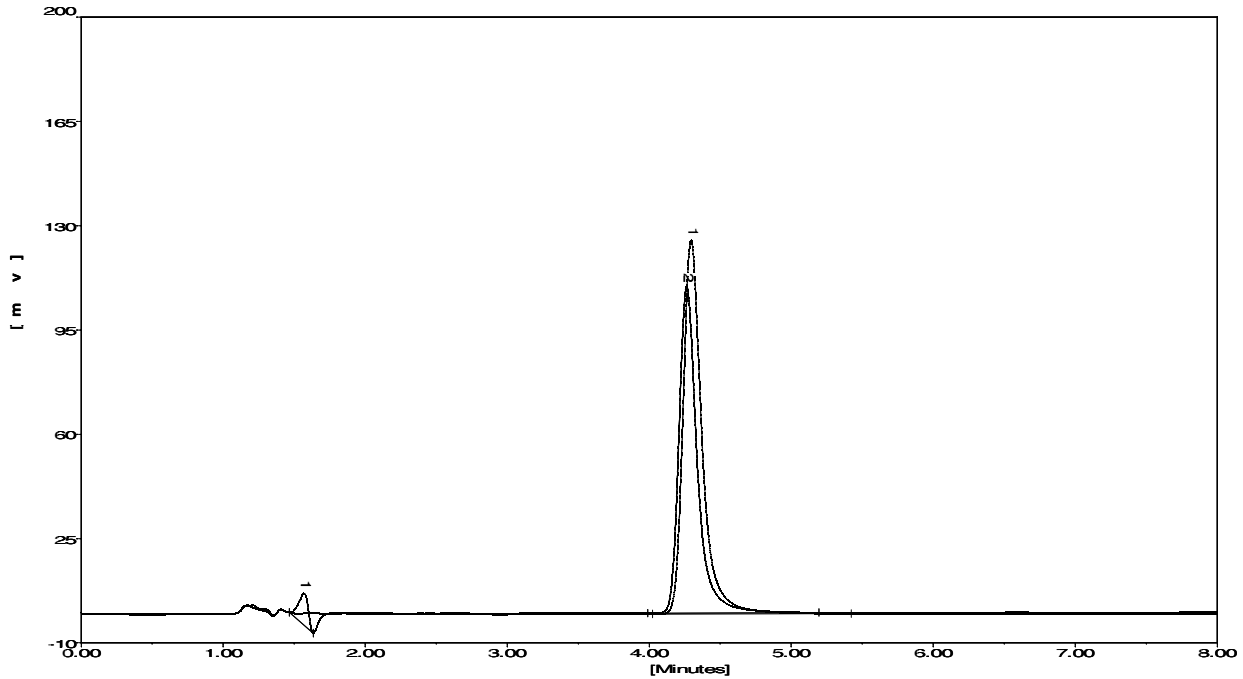
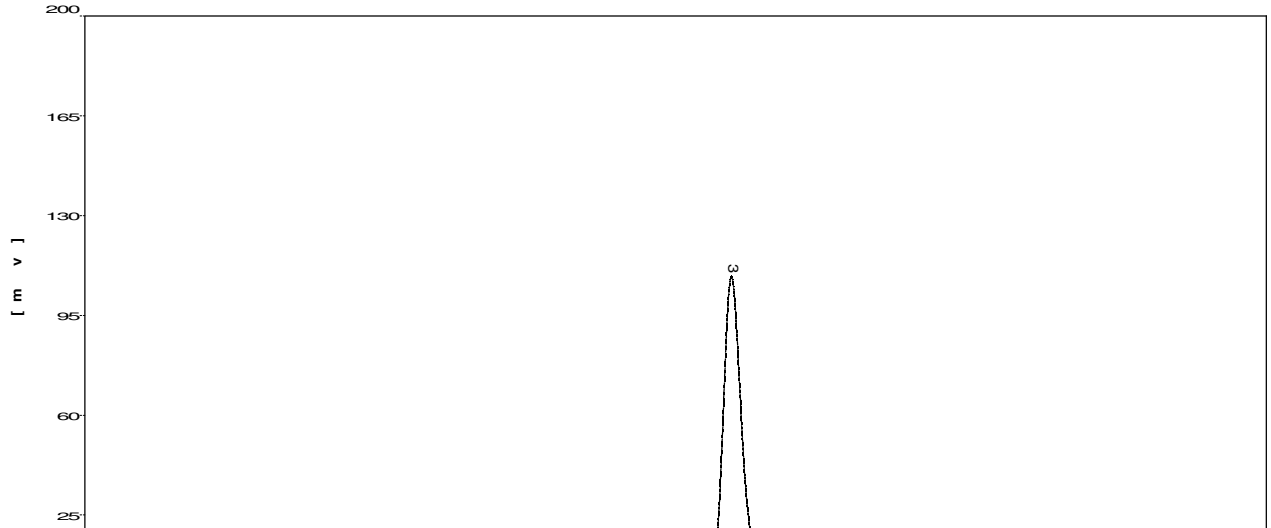


Figure: 4.21. Overlapping chromatogram of linagliptin treated with 0.1N HCl at 60ºC for 3 hours and standard linagliptin.



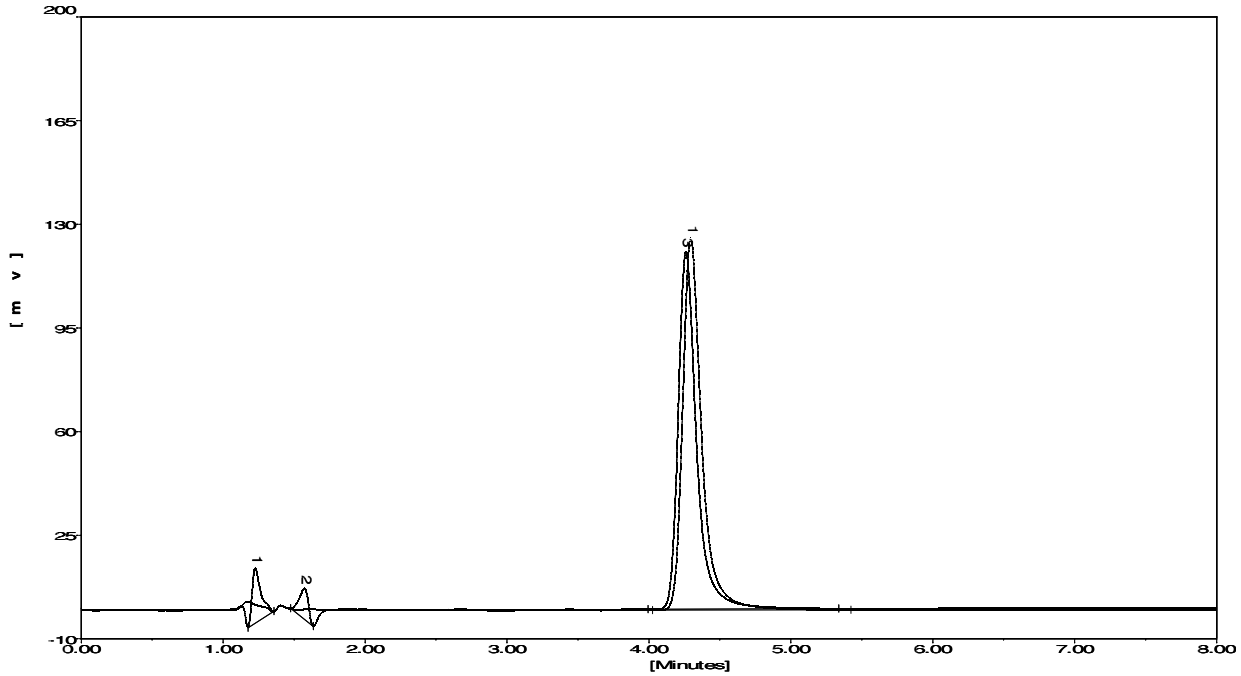
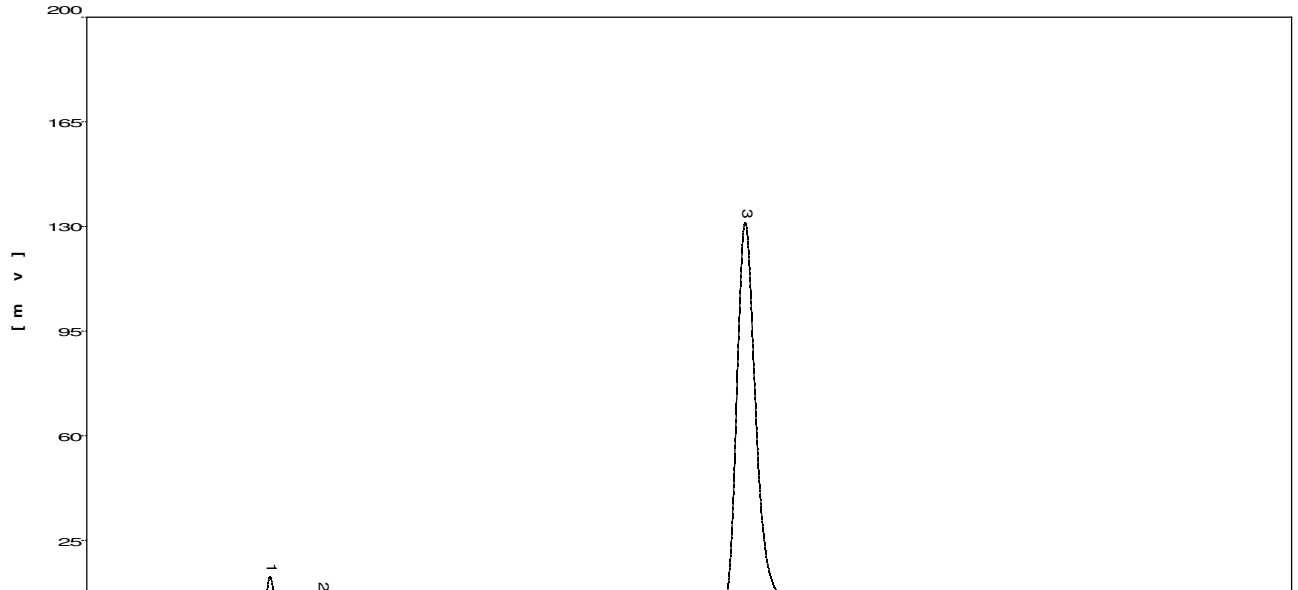


Figure: 4.23. Overlapping chromatograms of linagliptin treated with 1N HClat room temperature for 3 hours and standard linagliptin.



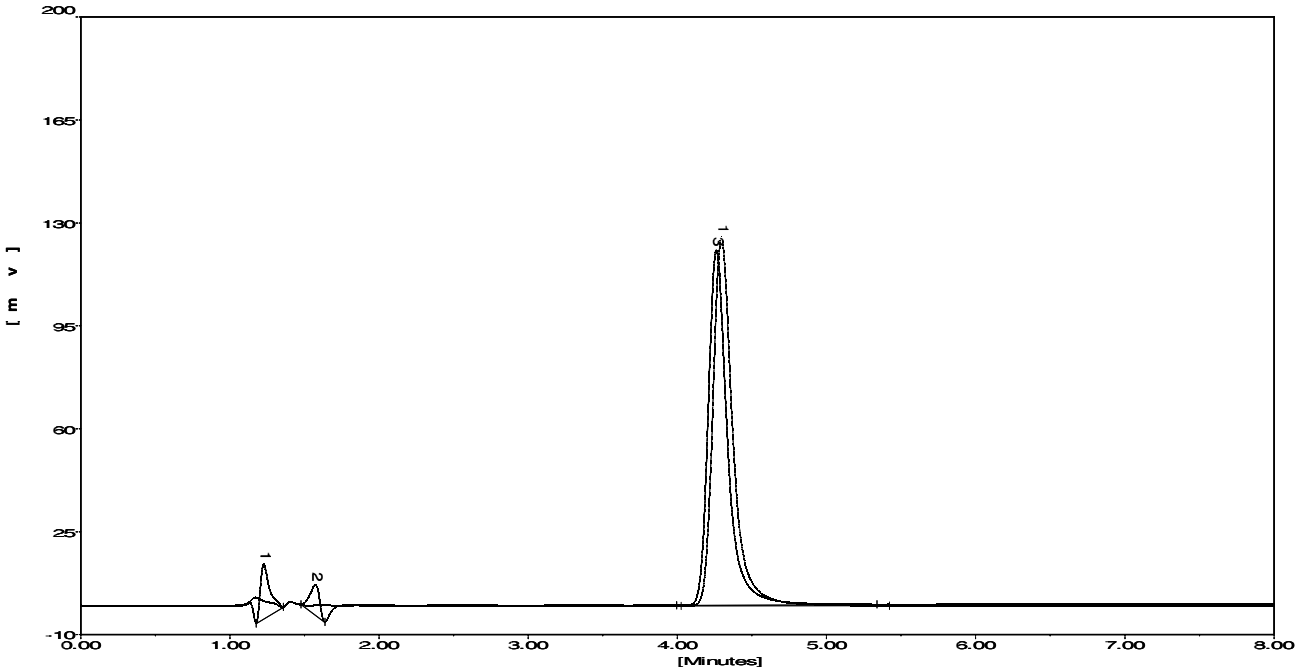
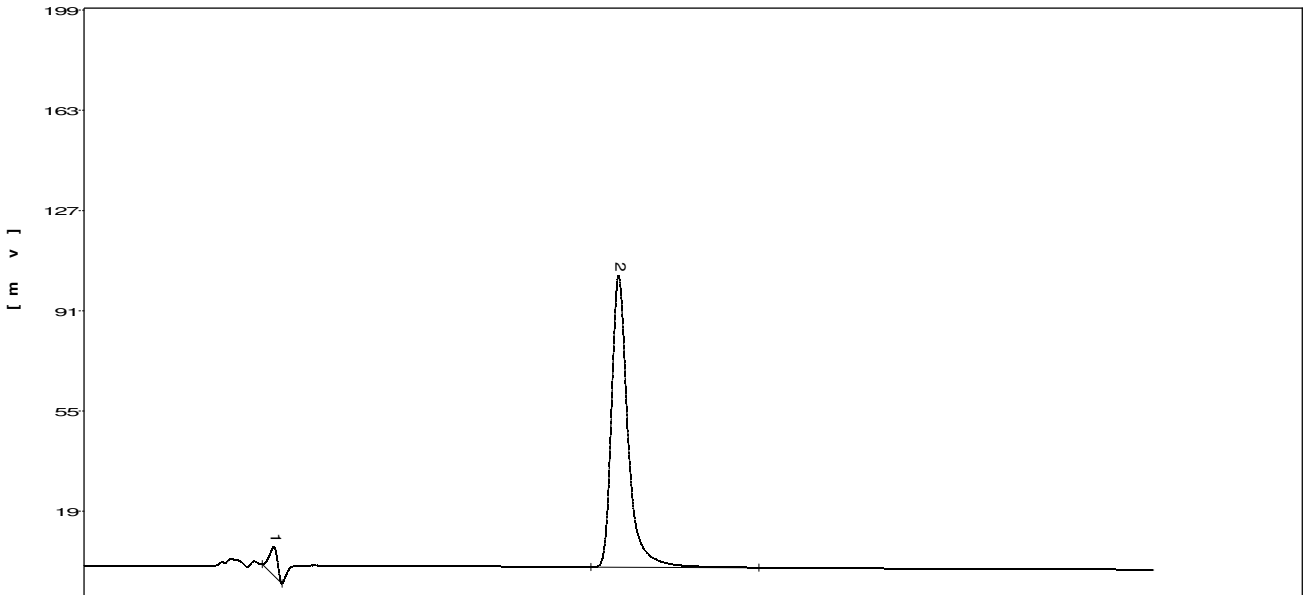


Figure: 4.25. Overlapping chromatograms of linagliptin treated with 1N HCl at 60ºC for 3 hours and standard linagliptin.



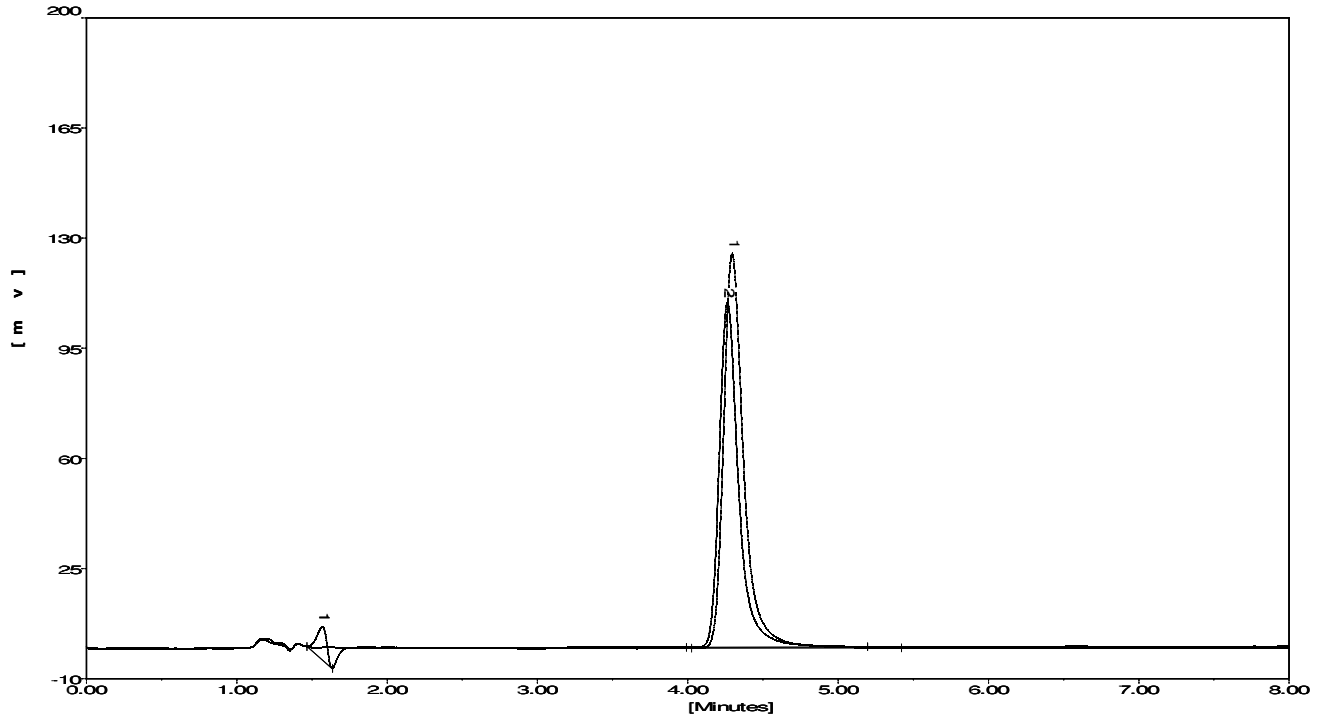


Figure: 4.27. Overlapping chromatograms of linagliptin treated with 0.1N HCl at 60ºC for 24 hours and standard linagliptin

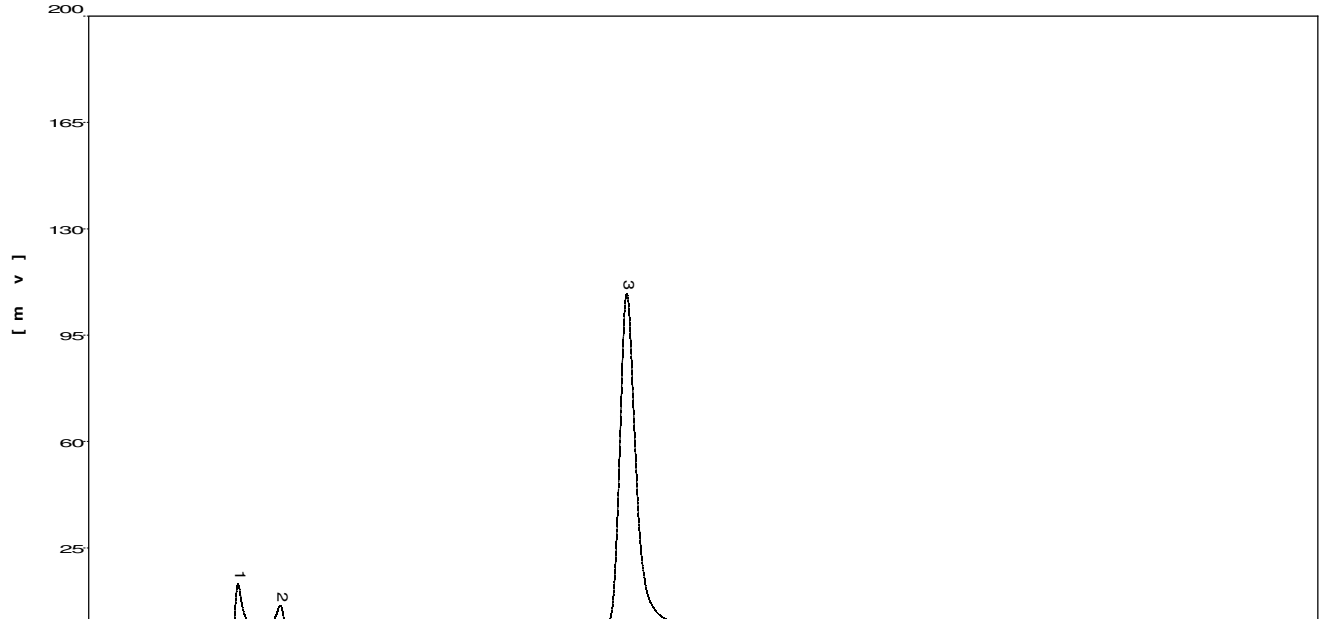


Figure: 4.28.Chromatogram of linagliptin treated with 1N HCl at room temperature for 24 hours

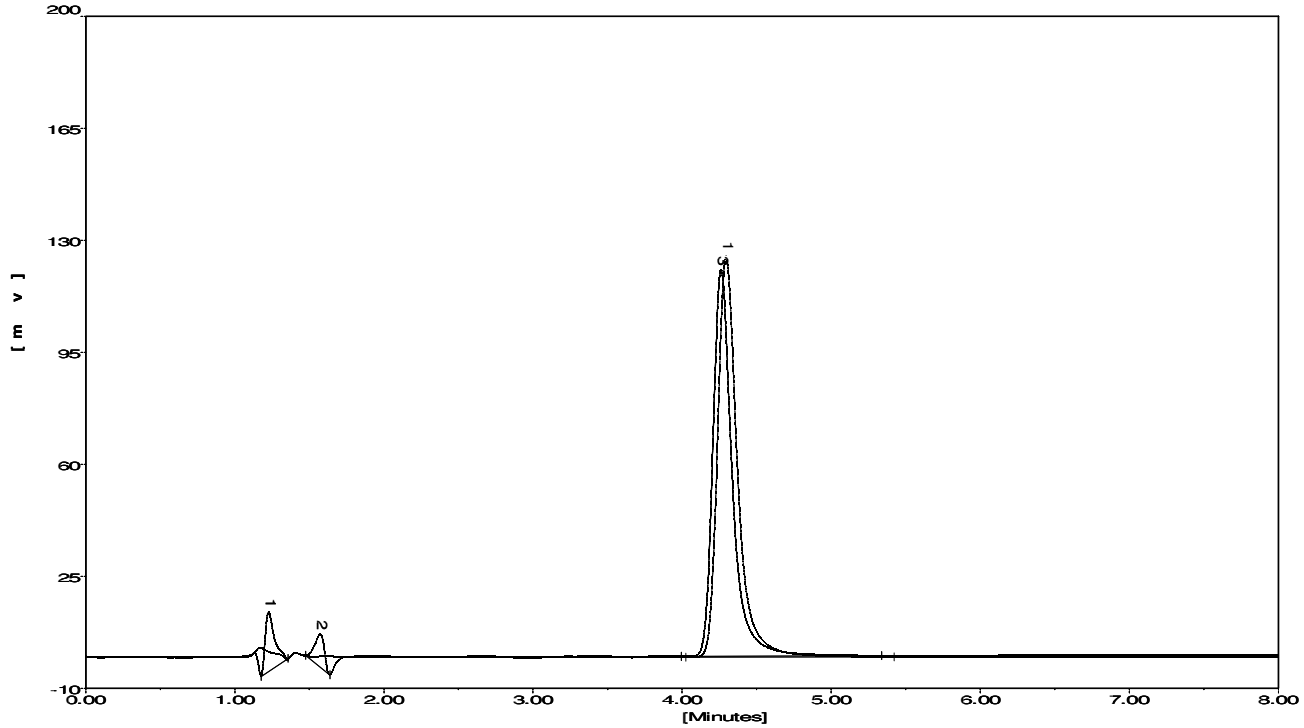


Figure: 4.29. Overlapping chromatogram of linagliptin treated with 1N HCl at 60ºC for 24 hours and standard linagliptin

BASE INDUCED DEGRADATION

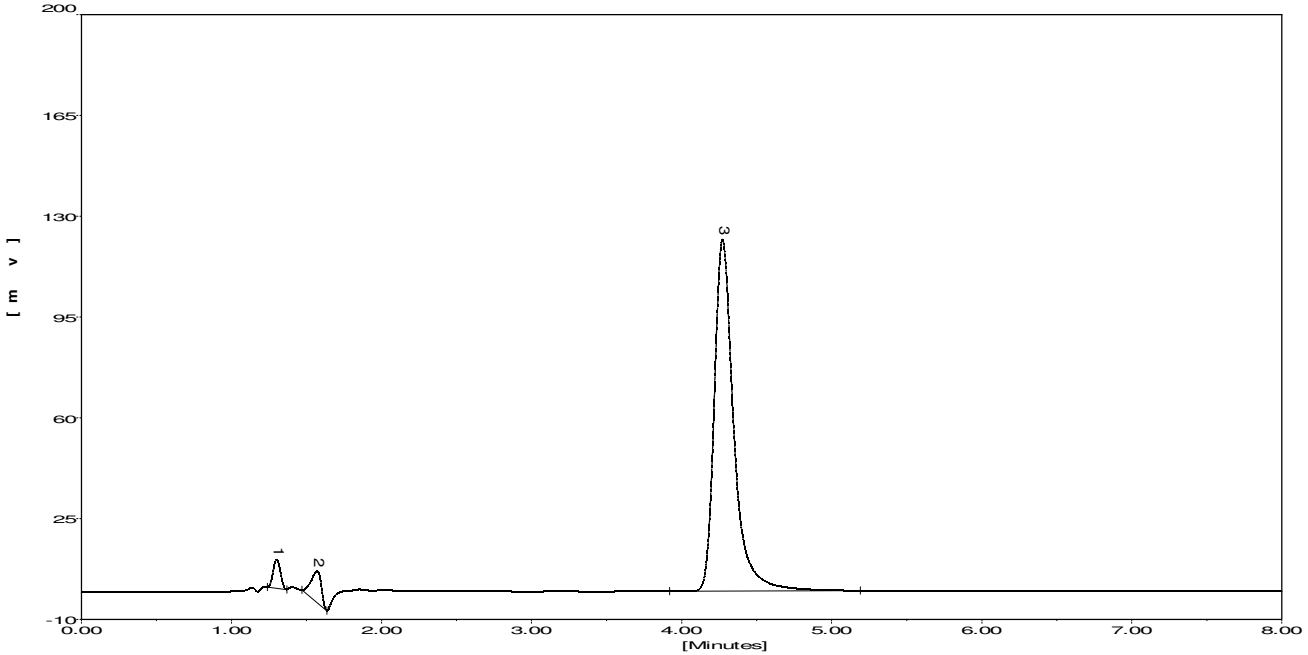
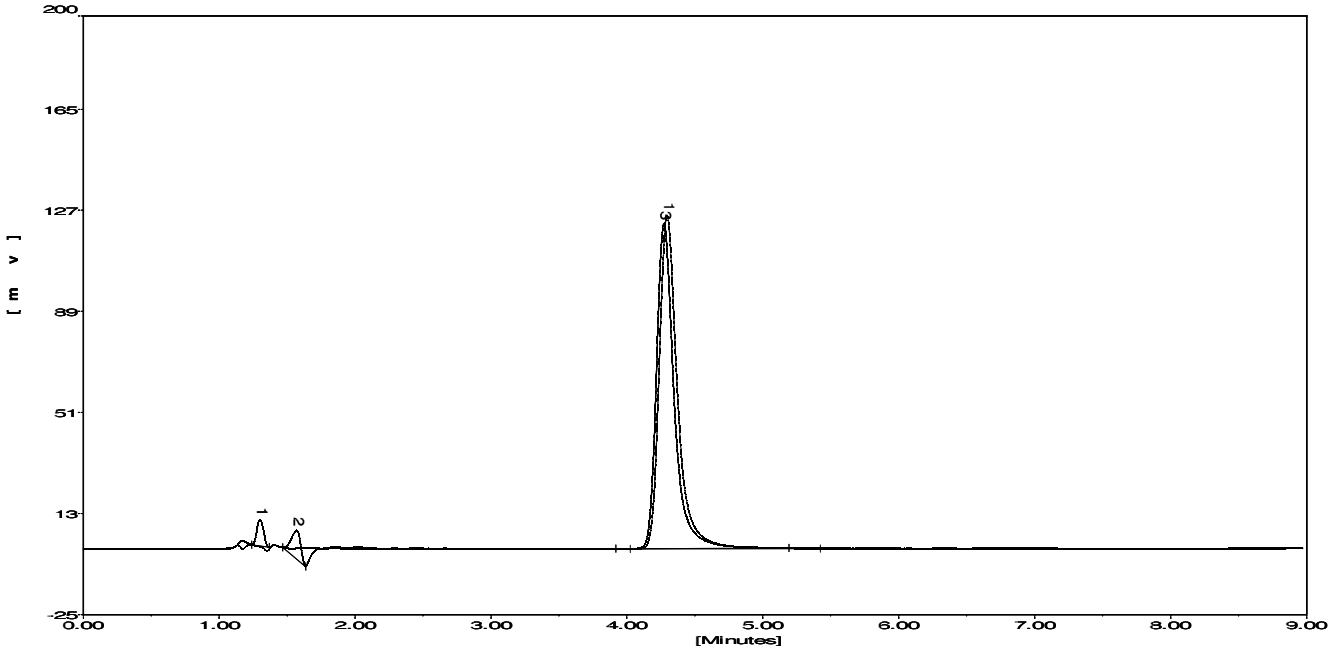


Figure: 4.30. Chromatogram of linagliptin treated with 0.1N NaOH at room temperature for 3hours



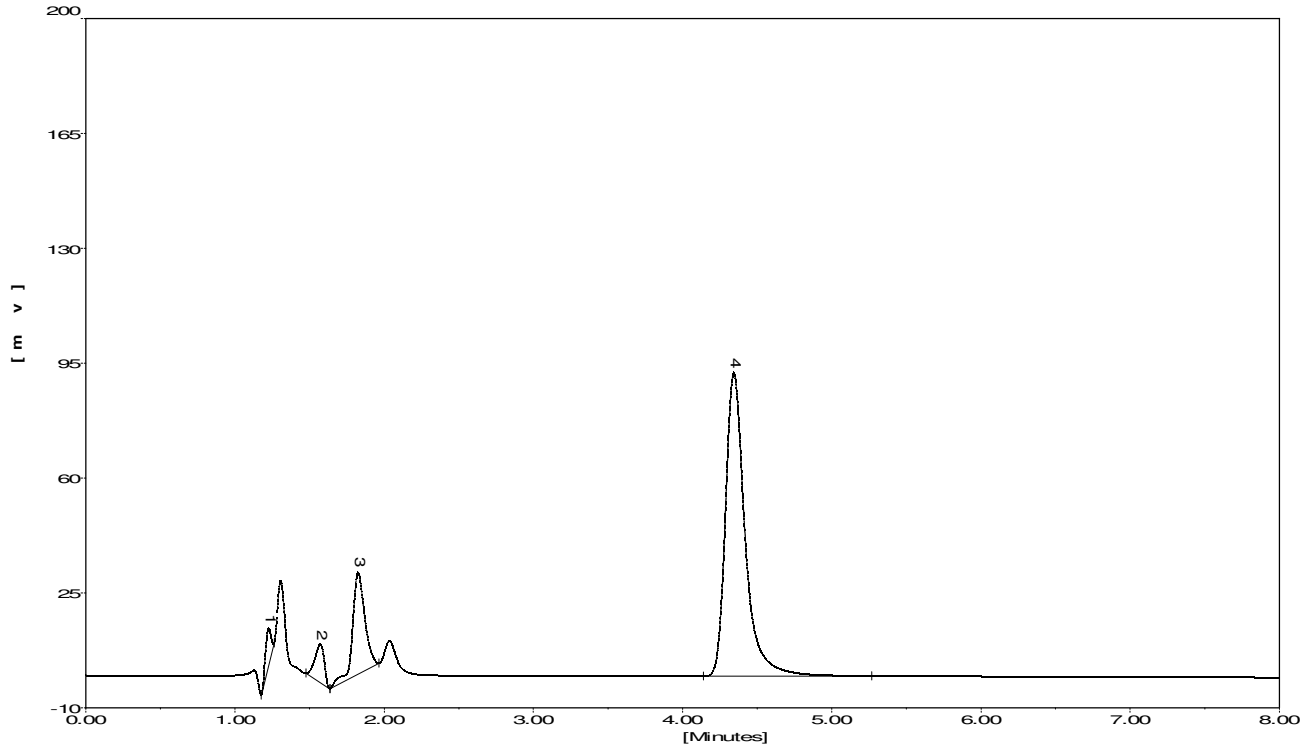
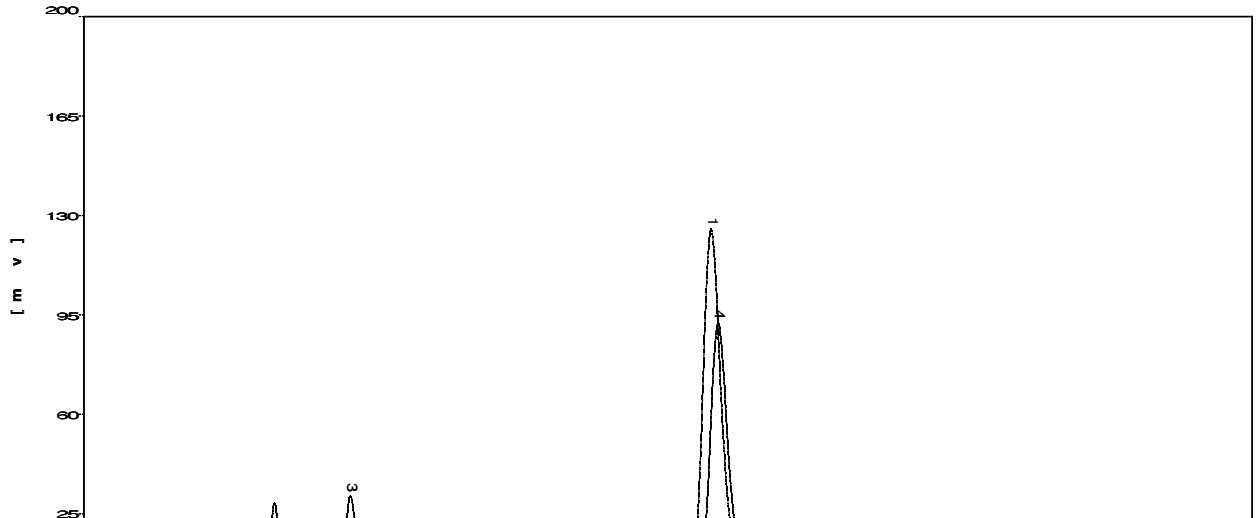


Figure: 4.32. Chromatogram of linagliptin treated with 1N NaOH at room temperature for 3hours



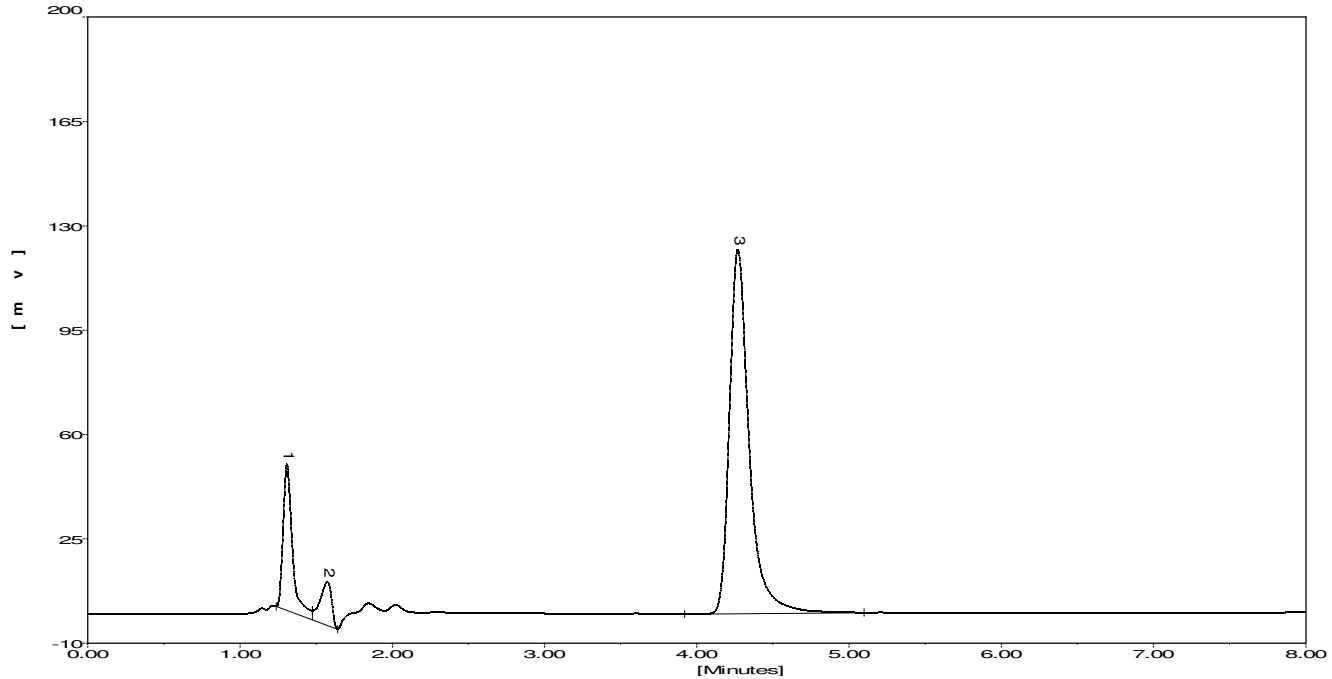
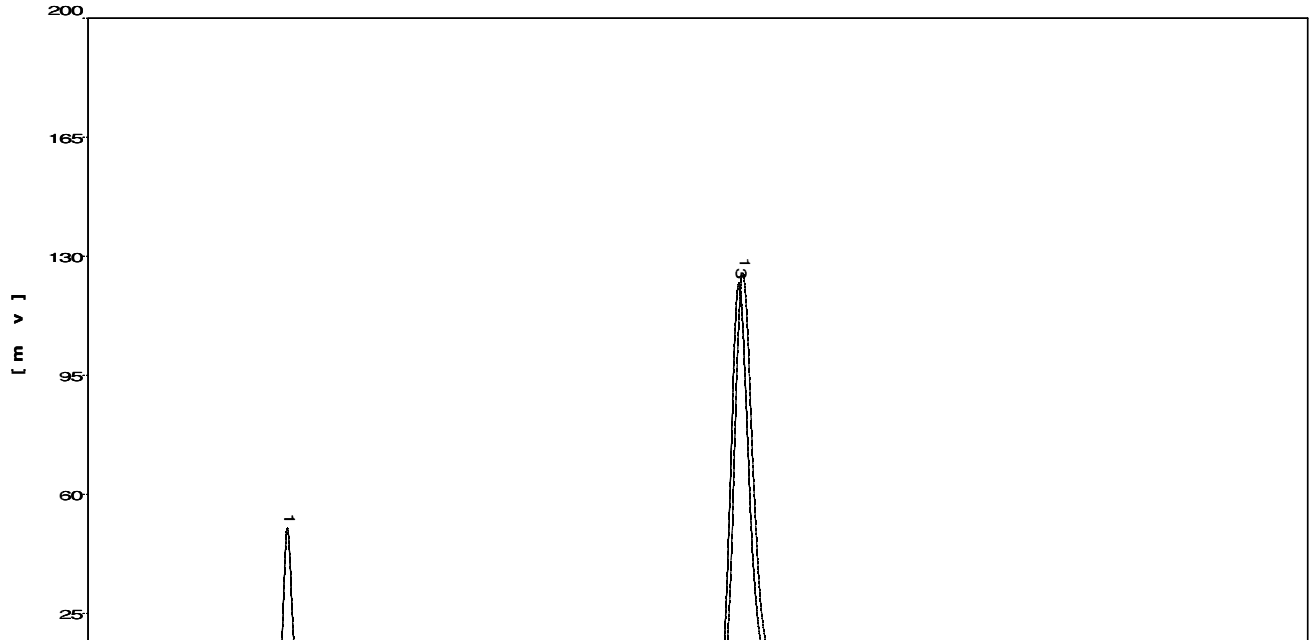


Figure: 4.34.Chromatogram of linagliptin treated with 0.1N NaOH at 60ºC for 3hours



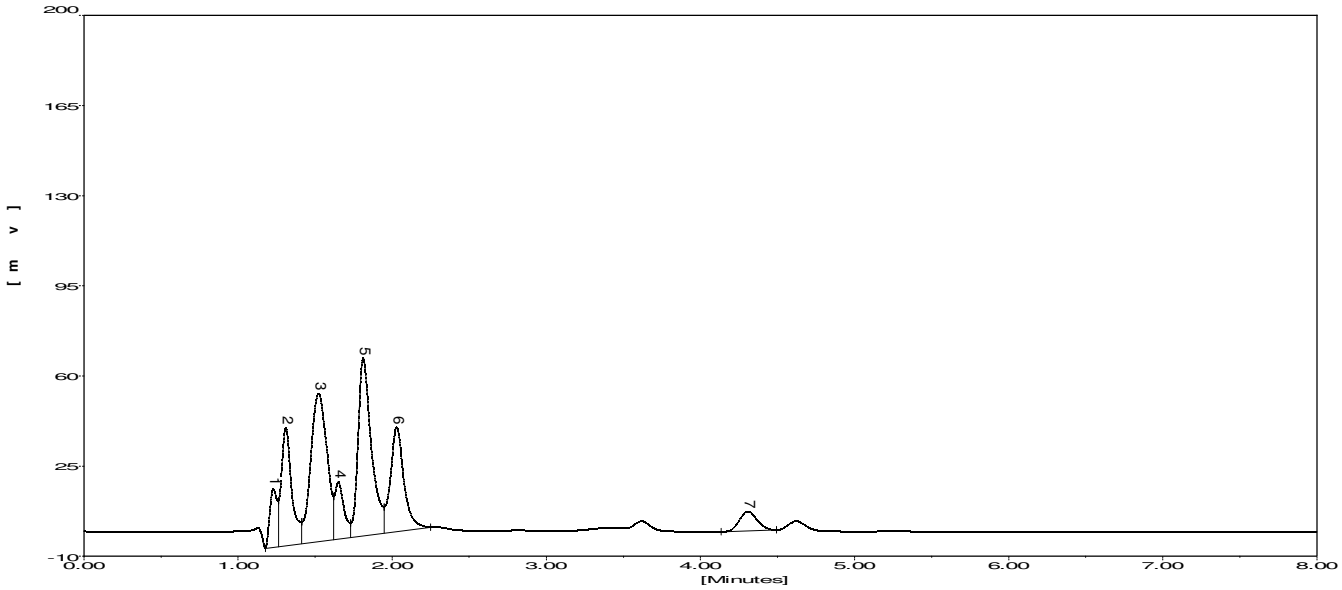
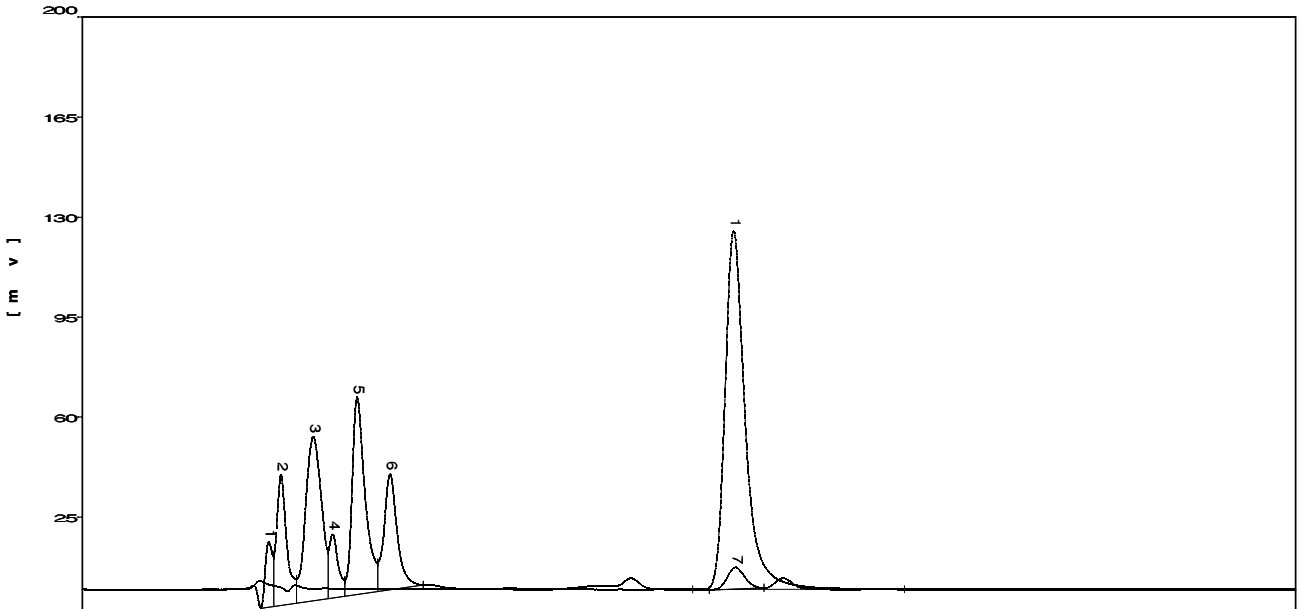


Figure:4.36. Chromatogram of linagliptin treated with 1N NaOH at 60ºC for 3hours

Peak area	Tailing factor	Retention time	Theoretical plates
59.79	1.21	4.31	226345



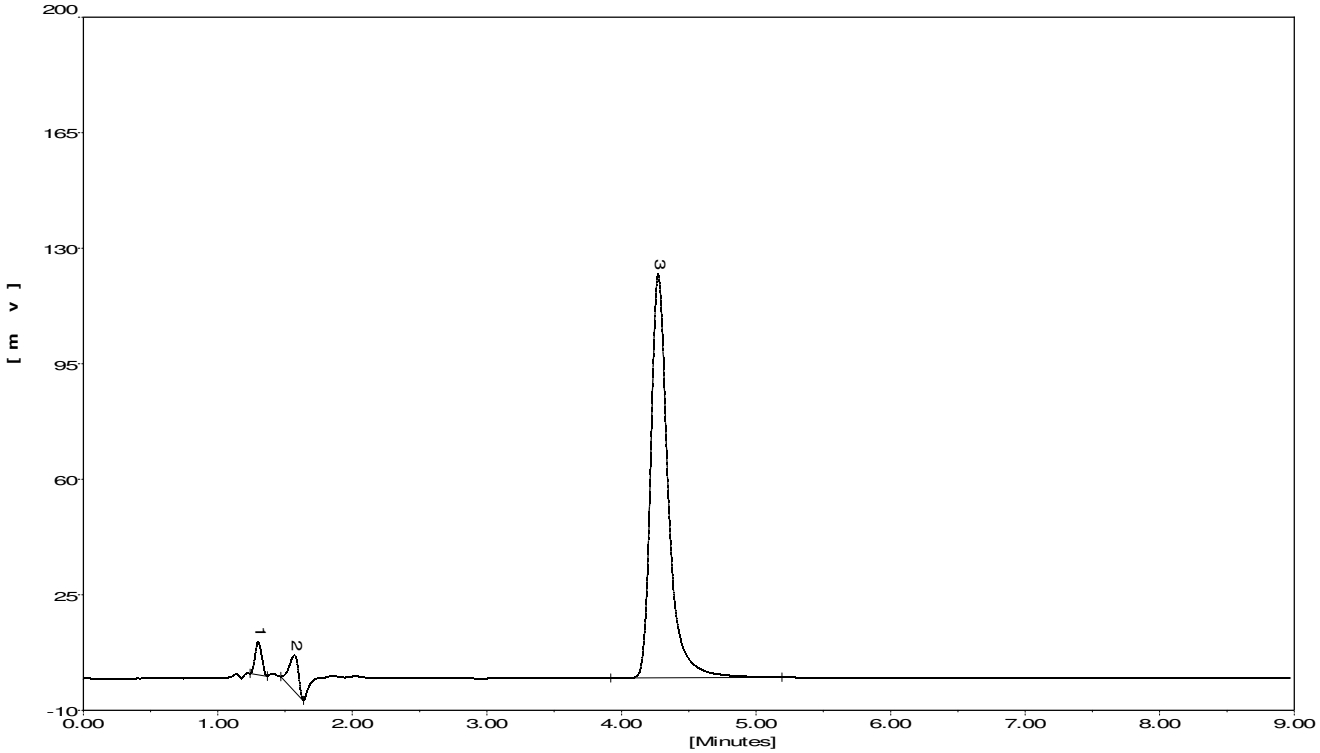


Figure: 4.38.Chromatogram of linagliptin treated with 0.1N NaOH at room temperature for 24hours.

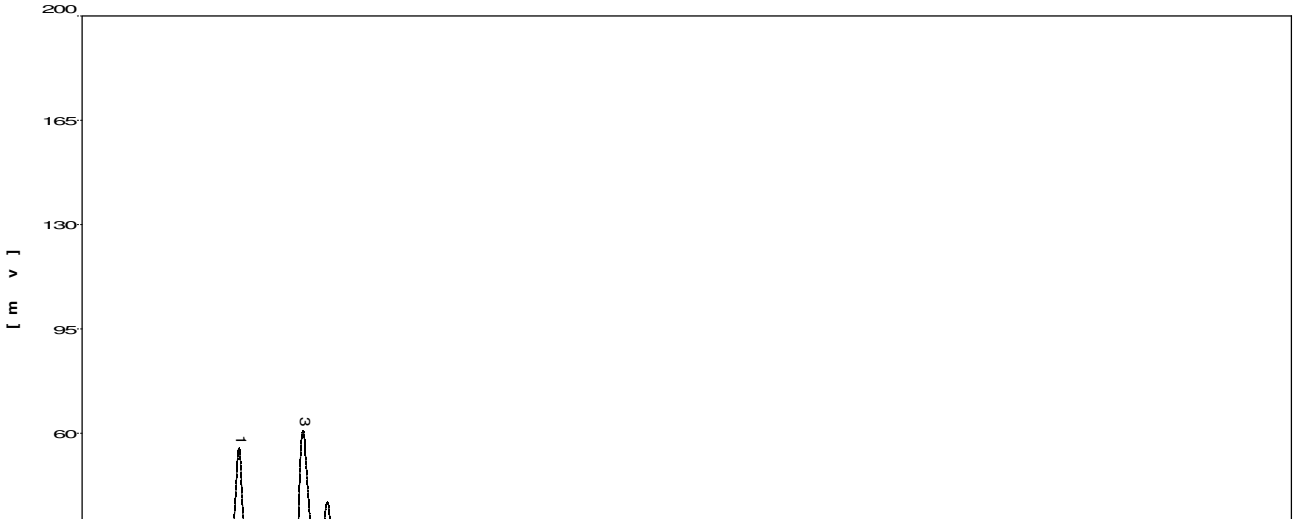
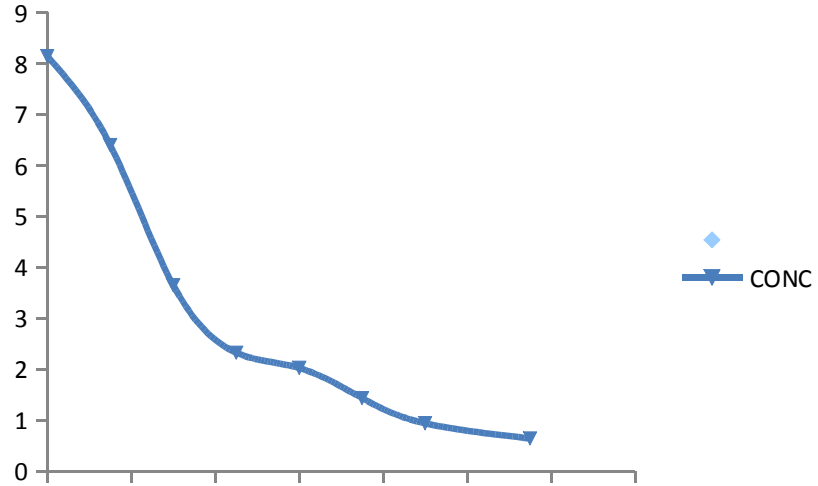


Table No 4.12. Resultsof linagliptin treated with 1N NaOH at 60°C at time intervals 0, 15, 30, 45, 60, 75, 90, 115min

TIME	Cocn X = (Y-c)/m
0	8.622183
15	6.399781
30	3.471725
45	2.052308
60	1.614316
75	1.241212
90	0.934374
115	0.770776



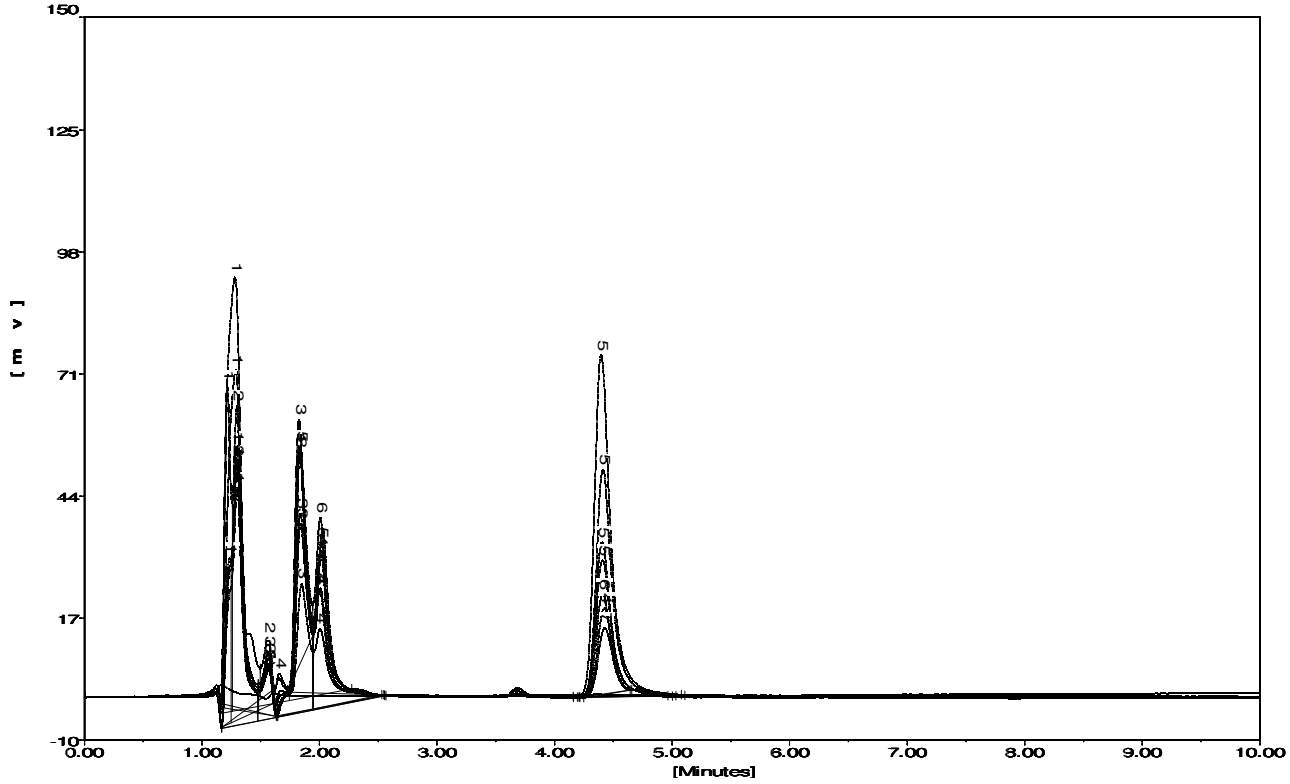


Figure: 4.41. Overlapping chromatogramsof linagliptin treated with 1N NaOH at 60ºC at time intervals 0, 15, 30, 45, 60, 75, 90, 115min

Table No 4.13. Resultsof linagliptin treated with 1N NaOH at 60ºC at time intervals 0, 3,6,9, 12, 15, 18 min

TIME	Cocn X = (Y-c)/m
0	8.143556
3	7.585846
6	7.421762
9	6.707997

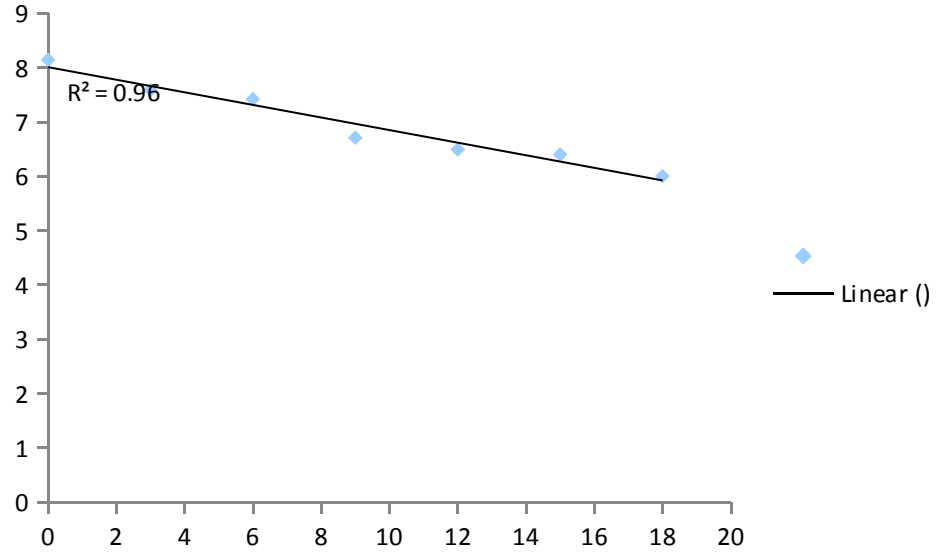
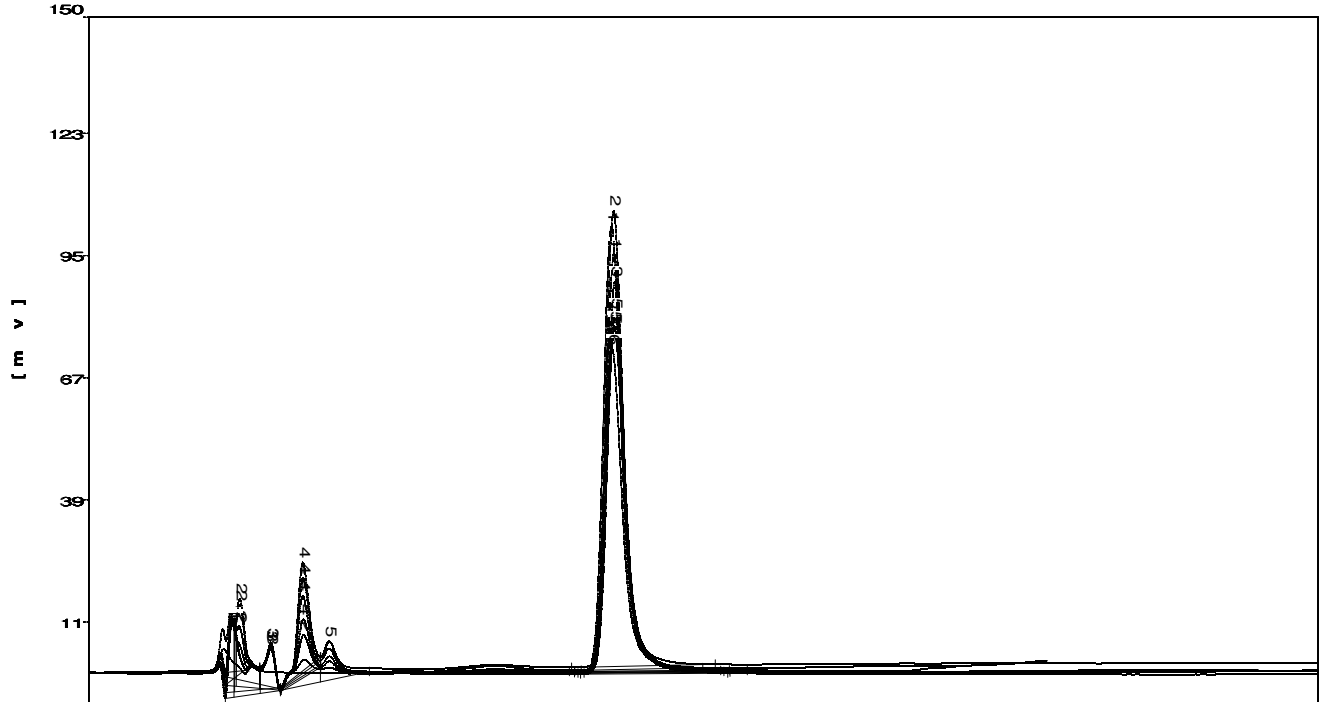


Figure: 4.42.Plot of Time vs Conc



5. SUMMARY

PRELIMINARY STUDY

DETERMINATION OF MAXIMUM WAVELENGTH (λ_{max})OF DRUG:

The maximum wavelength of absorption (λ_{max}) of linagliptin was established by scanning the drug solution (20µg/ml) for UV absorption. The absorption spectra showed two absorption maxima, at 225nm and 295nm.Further, calibration curve obtained by plotting absorption versus concentration was linear at 225 nm in 0.8 -8 µg/mL range with coefficient of determination of 0.996 and at 295 nm in 2-25µg/mL range with coefficient of determination of 0.992.

From the aforementioned data, the 225 nm was found to be appropriate wavelength for analysis. The data of scan spectra of linagliptin was shown in **Fig: 3.1.**

DETERMINATION OF LINEARITY IN UV:

Linearity was established by least squares linear regression analysis of the calibration curve. The constructed calibration curve was linear over concentration range of 2 to 25µg/mL at 295nm and 0.8 to 8 µg/mL at 225nm. Absorbance of linagliptin was plotted versus their respective concentrations and linear regression analysis on the resultant curves. Typically, the regression equation was y = 0.042x – 0.016 at 295nm and y = 0.123x + 0.027 at 225nm. The data of linearity was illustrated in **Table: 3.1 and Table: 3.2.**

METHOD DEVOLOPMENT AND OPTIMIZATION

The chromatographic method was optimized by changing various parameters such as pH of the mobile phase, organic modifier and composition of the mobile phase. The linagliptin UV absorption spectra revealed two absorption maxima at 225 nm and 295 nm. The response with wavelength 295 nm was low compared with 225 nm, which was selected as detection wavelength for analysis. Initially mobile phase used was 30 mM phosphate buffer (pH 3) and methanol (60:40, % v/v) gave bad peak shape, very less theoretical plates and retention time was too long. To improve these parameters,

acetonitrile was introduced into mobile phase, 30 mM phosphate buffer (pH 3), acetonitrile and methanol (60:30:10, % v/v) employed as mobile phase, LC analysis indicated broad peak shape and theoretical plates are below the acceptance criteria. Further methanol was eliminated from mobile phase, the binary solvent system consisting 30 mM phosphate buffer (pH 5), and acetonitrile (30:70, % v/v), produced no improvement in theoretical plates count. Further, theoretical plates were improved by increasing buffer concentration to 40 mM and keeping the pH same but peak tailing was more.Subsequently,pH of the buffer reduced to 3, approximately 1.1 units above pK_{a2} of linagliptin, acceptable theoretical plates count and peak tailing factor were obtained, probably due to less ionization of linagliptin imparting decreased polar contacts with stationery phase. Finally, using C8 (25 cms long 4.6mm inner diameter) column, mobile phase consisting of 40 mM phosphate buffer (pH 3), and acetonitrile (70:30, % v/v), at flow rate 1 mL/min produced all system suitability parameters in acceptable range.

METHOD VALIDATION:

SYSTEM SUITABILITY

System suitability tests are integral part of method development and are used to ensure adequate performance of the chromatographic system. The parameters of these tests are retention time (RT), number of theoretical plates (N) and tailing factor (T), were evaluated for six replicate injections of the drug at concentration 10 µg/mL. Mean ±SD, RSD (%) of retention time, capacity factor (k), theoretical plates (N) and tailing factor (T) for linagliptin was found to be 4.19 ± 0.03, 2.30 ± 0.002, 22364 ± 198, 1.52 ± 0.004 respectively. The data system suitability was illustrated in **Table: 3.3.**

LINEARITY

Linearity was established by least squares linear regression analysis of the calibration curve. Peak areas of linagliptin were plotted versus their respective concentrations and linear regression analysis on the resultant curves. The constructed calibration curve was linear over concentration range of 0.2 to 16 µg/ml. Typically, the regression equation was y = 125.8x + 17.58, and correlation coefficient was 0.999, illustrates good linearity of

calibration curve having high correlation coefficient. The data of linearity was illustrated in **Table: 3.4.,** and the linearity curve was plotted and given in **Fig: 3.6.**

ACCURACY

Accuracy of the method was determined by performing the % recovery experiment. Known amount of standard solution at 80, 100 and 120% levels were fortified to the formulation solution. Peak areas of standard were calculated by the difference of peak areas between fortified and unfortified samples. Three replicate samples of each concentration level were analysed and the % recovery at each level (n=3), and mean % recovery (n=9). For, linagliptin the results obtained were in good agreement with label claim. The results reported in **Table 3.6.**

SPECIFICITY:

The specificity of the HPLC method was determined by the complete separation of linagliptin along with other parameters like retention time (RT), capacity factor (k), tailing or asymmetrical factor (T), etc. The results obtained were shown in **Fig: 3.8.** and**Fig: 3.9.**Mean retention time (min) **±** SD for linagliptin of concentration 10µg/mL was found to be 4.18 ± 0.0008, 0.19 respectively, for six replicates of standard solution. The peaks obtained were sharp and have clear baseline separation. The chromatogram of the pharmaceutical formulation indicated that no additional peaks and interference from any excipients. In addition, degradation studies showed good resolution and devoid of interference from any of the degraded products.

PRECISION

Method precision

The intra and inter-day variability or precision data and were assessed by using standard solutions prepared to produce solutions of three different concentrations of drug (6, 10 and 14 µg/mL), representing 60, 100, 140% respectively. Repeatability or intra-day precision was investigated by injecting three replicate samples of each of the samples of three different concentrations. Inter-day precision assessed by injecting the same three concentrations over three consecutive days. The % RSD results of repeatability and

intermediate precision were within 2.0%; ascertaining good precision of the proposed new method. The results were reported in **Table: 3.8.**

Intra-day precision

Mean peak area (mv.sec) **±** SD,%RSD for linagliptin of concentrations 6, 10 and 14 µg/mL are found to be 796.66 ± 0.015,1.56 ; 1289 ± 0.05, 0.50 and 1770 ± 0.049,0.35 respectively. Mean retention time **±** SD,%RSD for linagliptin of concentrations 6, 10 and 14 µg/mL are found to be 4.24 ± 0.005 , 0.13; 4.26 ± 0.025, 0.59 and 4.24 ± 0.02, 0.49 respectively. The results were reported in **Table: 3.8**

Inter-day precision

Mean peak area **±** SD,RSD(%) for linagliptin of concentrations 6, 10, and 14 µg/mL are found to be 801 ± 0.133, 1.17; 1283.11 ± 0.06677, 0.66and 1770.66± 0.067, 0.48 respectively. Mean retention time **±** SD,%RSD for linagliptin of concentrations 6, 10 and 14 µg/mL are found to be 4.24 ± 0.005, 0.12; 4.26 ± 0.022, 0.527 and 4.24 ± 0.015, 0.354 respectively. The results were reported in **Table: 3.8.**

SYSTEM PRECISION

The intra and inter-day variability or precision data and were assessed by using standard solution of concentration 10µg/mL. Repeatability or intra-day precision was investigated by injecting 10µg/mL for six times within the same day. Inter-day precision assessed by injecting 10µg/mL for six times over three consecutive days. Both intraday precision and interday precision of the new analytical method are found to be good based on %RSD, corresponding to peak areas and retention times.

Intra-day precision

Mean peak area (mv.sec) **±** SD, % RSD for linagliptin of concentration 10µg/mL was found to be 1295 ± 18.8, 1.45, mean retention time (min) **±** SD, % RSD for linagliptin of concentration 10µg/mL was found to be 4.18 ± 0.0008, 0.19. The results were reported in **Table: 3.7.**

Interday precision

Mean peak area (mv.sec) **±** SD, % RSD for linagliptin of concentration 10µg/mL was found to be 1261 ±16.69, 1.32. Mean retention time (min) **±** SD, % RSD for linagliptin of concentration 10µg/mL was found to be 4.22 ± 0.03, 0.787respectively. The results were reported in **Table: 3.7.**

SOLUTION STABILITY

The stability of linagliptin in mobile phase was investigated by analyzing the standard of linagliptin (10µ/mL) at 0min, 15min, 30min, 1hr, 2hrs, 3hrs, 24hrs, after preparation at room temperature. No significant variation in the peak area of standard solution was observed up to 24 hours, also no additional peak were found in the chromatogram revealing stability of linagliptin in mobile phase. The results were reported in **Table: 3.11** and chromatogram of solution stability were represented as **Fig: 3.11. and 3.12.**

ROBUSTNESS

The robustness was determined by deliberately modifying the experimental conditions. The parameters varied are flow rate (±10%), organic strength (±7%) and buffer concentration (±12%).

(i)Effect of variation in flow rate:

Mean ±SD (n=6) of retention time (min), tailing factor and peak area (mv.sec) for linagliptin was found to be 4.47 ± 0.29, 1.41 ± 0.011 and 1229.697 ± 32.12 respectively. The results were reported in **Table: 3.10.**

(ii)Effect of variation in mobile phase composition (acetonitrile):

Mean± S.D (n=6) of retention time (min), Tailing factor and Peak area (mv.sec) for linagliptin were 4.52 ± 0.45, 1.29±0.02 and 1221.33 ± 32.72 respectively. The results were reported in **Table 3.10.**

(iii)Effect of variation of buffer pH in mobile phase:

Mean ± S.D (n=6) of retention time (min), tailing factor and peak area (mv.sec) for linagliptin was found to be 4.48 ± 0.055, 1.42 ± 0.2 and 1230.33 ± 26.274 respectively. The results were reported in **Table: 3.10.**

(iv)Effect of variation in buffer concentration:

Mean± S.D (n=6) of retention time (min), tailing factor and peak area (mv.sec) for linagliptin was found to be 4.47 ± 0.05529, 11.42 ± 0.01, 1241.66 ± 18.50 respectively. The results were reported in **Table 3.10.**

The results showed that, none of the significantly influenced the tailing factor and theoretical plates. The retention time was un affected by change in buffer pH, concentration, although minor change were found in flow rate and organic strength variation, but separation, quantification was not influenced.

LOD & LOQ

Lowest amount of analyte that can be detected is limit of detection but not necessarily to be quantified. Lowest amount of analyte that can be detected and is quantified is limit of quantification. They are determined by performing three sets of calibration curve of concentrations ranging from 6-16µg/mL, subsequently calculated by using standard deviation of y interceptand mean of the slope.LOD is found to be 0.008µg/mLand LOQ was found to be 0.025µg/mL. Over all the analytical method was very sensitive. The results were reported in **Table 3.5.**

ASSAY

The validated method was applied was to the determination of linagliptin in commercially available Trajenta tablets. The result of the assays (n=3) undertaken yielded 101% of label claim for linagliptin. The observed concentration of linagliptin was found to be 5.05mg, which is in good agreement with label claim.The results were shown in **Table: 3.9**.

THE FORCED DEGRADATION STUDIES

Stress studies were carried out following an ICH guideline which establishes the requirements of stability indicating methods. Stress testing provides evidence on how the quality of a drug may be affected under the influence of different stress conditions. Drug decomposition may result in loss of potency and advent of possible adverse effects due to the formation of degradation products. The tests were performed on 1mg/mL solution of linagliptin using various conditions acidic, basic and oxidizing conditions. Thermal condition was performed on 10mg of linagliptin solid drug for 10 days at 60ºC.

ACID INDUCED DEGRADATION:

Analysis of chromatograms of the acidic degraded samples for linagliptin using 0.1N HCl either at room temperature or at 60ºC temperature demonstrated additional peak at 1.61 min. employing 1N HCl for degradation either at room temperature or at 60ºC temperature showed additionalpeaks at 1.21 min and 1.62 min. Further, estimation of retention times and peak areas illustrated that peaks of degraded products were resolved from the drug peak and no significant degradation in every condition up to 24hours. The results were shown in the **Fig: 3.18, 3.19, 3.20, 3.21, 3.22, 3.23, 3.24, 3.25, 3.26, 3.27, 3.28 and 3.29.**

THERMAL DEGRADATION:

The linagliptin degraded under dry heat conditions showed no additional peaks, this was confirmed by good peak area. This indicates linagliptin is resistant to dry heat degradation.The results were shown in theare shown in **Fig: 3.14 and 3.15.**

OXIDATIVE DEGRADATION:

The linagliptin degraded with 3% hydrogen peroxide showed no additional peaks, depicting linagliptin resistance to oxidative degradation.The results were shown in the **Fig: 3.16 and 3.17.**

BASE INDUCED DEGRADATION:

Preliminary base induced degradation revealed linagliptin stability is not significantly effected by treatment with 0.1N NaOH up to 24 hours at room temperature and 3 hours at 60ºC. In these conditions two impurity peaks were observed at 1.3 min and 1.53 min, were resolved from the drug peak. Further treatment with 1N NaOH for 24 hours at room temperature resulted in significant degradation, approximately 86% of the drug degraded. Conducting similar study at higher temperature (60ºC), near complete degradation was observed at 3 hours. The impurities formed, at room temperature eluted at 1.31min and 1.62min retention time, impurities formed at 60ºC eluted at 1.2 to 2.1 min retention time. Interestingly none of them interfered with drug peak.The results were shown in the **Fig: 3.30, 3.31, 3.32, 3.33, 3.34, 3.35, 3.36, 3.37, 3.38 and 3.39**. Over all summary of base degradation was shown in the **Table: 3.12.**

In addition, to know more about 1N NaOH induced degradation at 60ºC, two studies were conducted. First study duration was 115 minutes, samples were analyzed at every 15 minutes interval. The results obtained are shown in **Table: 3.13. and Fig:3.43.** Second study was conducted for 18 minutes duration, samples were analyzed at every 15 minutes interval.The results obtained are shown in **Table: 3.14. and Fig:3.47.** Both the studies showed time dependant degradation of linagliptin.

6. CONCLUSION

The present study describes sensitive and reproducible method for the quantitative determinationlinagliptin and its degradation behavior. The method was based on high performanceliquid chromatographic separation of the drug on the MicrosorbC8 (25 cms long 4.6mm inner diameter)column at ambient temperature using mobile phase potassiumdihydrogen phosphate(40mM: pH 3) and acetonitrile (70:30, v/v). Flow rate was 1.0mL/min and retention time was found to be 4.19min. Quantitation was achieved with UV detection at 225nm based on peak area with linear calibration curve at concentration range 0.2 - 16µg/mL. This method has been successively applied to pharmaceutical formulation. No chromatographic interference from the tablet excepients was found. The method was validated. Relative standard deviation values for all key parameters were less than 2.0%. Excellent recoveries (98.51 and 99.82%) proved that the method was sufficiently accurate. The LOD and LOQ were found to be 0.06 and 0.18µg/mL, respectively. The RSD valves for intraday and inter-day precision below 2.0%. Acceptable robustness indicates that the assay method remains unaffected by small but deliberate variations.

Further more, developed method was suitable to study the degradation behavior of linagliptin under acid, alkali, oxidation and thermal conditions. The linagliptin was found to be degraded in alkaline conditions in time dependant manner. Future studies aiming on isolation and characterization of formed impurities may provide further insights.

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